

Open Research Online

The Open University's repository of research publications and other research outputs

Combination treatments in *in vitro* and *in vivo* models between molecules reverting epigenetic gene silencing and DNA-interacting anticancer drugs

Thesis

How to cite:

Sabatino, Maria Antonietta (2008). Combination treatments in *in vitro* and *in vivo* models between molecules reverting epigenetic gene silencing and DNA-interacting anticancer drugs. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2008 The Author



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.21954/ou.ro.0000eb08>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

COMBINATION TREATMENTS IN *IN VITRO* AND *IN VIVO* MODELS BETWEEN MOLECULES REVERTING EPIGENETIC GENE SILENCING AND DNA-INTERACTING ANTICANCER DRUGS

Thesis submitted for the degree of Doctor of Philosophy at the Open University

Discipline of Life Sciences

By

Maria Antonietta Sabatino, Degree in Biological Science

Mario Negri Institute for Pharmacological Research, Milan, Italy

March 2008

,



CKNOWLEDGMENTS

I would like to thank my supervisor, Dr Massimo Brogginì, Head of Molecular Pharmacology Laboratory, for his stimulating scientific discussion on my PhD project; I am grateful to Dr Giovanna Damia, director of studies, for her support and very precious suggestions. I would like to thank my external supervisor, Prof. Robert Brown, now at Imperial College in London, who gave me the opportunity to work in his laboratory at the Beatson Institute in Glasgow. I would thank Dr Cristina Geroni at Nerviano Medical Sciences for the stimulating discussion on the results obtained and for supporting my study on brostallicin.

I would especially thank the colleagues who helped me in performing some experiments: Jens Teodoridis and Kim Appleton at the Beatson Institute for teaching me new techniques on DNA methylation analysis, Roberta Ceruti for immunohistochemistry experiments and Prof. Anna Maria Caccuri at University of Tor Vergata in Rome for the kinetic studies.

A special thank to all colleagues of the Department of Oncology at Mario Negri Institute, who share with me the exciting experience of cancer research.

ABSTRACT

Epigenetic transcriptional gene silencing plays a fundamental role in cancer development and has been considered as a target for cancer therapy in the last few years, mainly due to its reversibility by small molecules. Among the several methylated genes investigated, glutathione-S-transferase (GST) P1, a protein belonging to cellular detoxification systems, has been shown to be extensively promoter-methylated in prostate cancer. My study therefore describes a new therapeutic approach against prostate cancer, based on the combination of demethylating agents and brostallicin, a DNA minor groove binding drug, which is activated in the cell by binding to glutathione, a reaction catalyzed by GST.

Among the demethylating molecules tested on the prostatic cancer cell line LNCaP *in vitro* combinations with brostallicin, zebularine was able to increase brostallicin activity with little toxicity compared to the other tested demethylating drugs.

These *in vitro* results prompted the *in vivo* testing of zebularine with brostallicin on LNCaP cells transplanted in mice. Prolonged treatment with zebularine was able to significantly improve brostallicin antitumour activity compared to both drugs administrated as single agents. When GSTP1 expression was investigated in treated samples *versus* untreated controls, no protein re-expression was found and this was related to the unchanged levels of GSTP1 promoter methylation. In contrast, the demethylating effect of zebularine was clearly evident in the promoter of GSTM1 gene, which is also silenced by methylation in LNCaP cells. GSTM1 codes for a class of GST enzymes that has recently been found to be more active on brostallicin than GSTP1.

This indicates that the activation of brostallicin cytotoxicity in LNCaP cells by zebularine likely depends on enzymatic activation by GSTM1 rather than GSTP1 and strengthens the feasibility of this combination as a treatment for prostate cancer in the clinic, and as model for the therapy of other solid tumours.

CONTENTS

| | |
|--|----|
| ACKNOWLEDGMENTS | 2 |
| ABSTRACT..... | 3 |
| CONTENTS..... | 4 |
| List of figures | 9 |
| List of Tables..... | 11 |
| 1. INTRODUCTION | 12 |
| 1.1 THE EPIGENETIC CODE..... | 13 |
| 1.1.2 DNA methylation..... | 14 |
| 1.1.3 DNA methyltransferases and Methyl Binding Proteins..... | 18 |
| 1.1.4 Histone Code..... | 20 |
| 1.1.5 Nucleosome Remodeling | 22 |
| 1.2 EPIGENETICS AND HUMAN DISEASES | 24 |
| 1.2.1 Imprinting disorders | 24 |
| 1.2.2 Epigenetics and Cancer | 27 |
| 1.3 PROSTATE CANCER | 38 |
| 1.3.1 Prostate Cancer: general characteristics..... | 40 |
| 1.3.2 Prostate Cancer and Epigenetic Modification..... | 42 |
| 1.4 GLUTATHIONE-S-TRANSFERASE | 50 |
| 1.4.1 GST enzymes | 50 |
| 1.4.1.1 Enzyme structures | 51 |
| 1.4.1.2 GSTpi hypermethylation..... | 54 |
| 1.4.1.3 Besides the GSH transfer catalysis: regulatory functions of GSTmu and pi | 57 |
| 1.4.1.4 Mitochondrial and MAPEG enzymes | 58 |
| 1.4.2 GSH..... | 59 |

| | |
|--|-----|
| 1.4.3 GST and CANCER | 60 |
| 1.5 ANTICANCER THERAPY | 63 |
| 1.5.1 Systemic Treatment for Prostate Cancer..... | 64 |
| 1.5.2 GST as a target for anticancer therapies | 67 |
| 1.5.3 Molecules Reverting Epigenetic Modifications of DNA..... | 69 |
| 1.5.3.1 Nucleoside inhibitors | 72 |
| 1.5.3.2 Zebularine | 76 |
| 1.5.3.3 Non nucleoside analog inhibitors of methylation | 79 |
| 1.5.3.4 HDAC inhibitors | 82 |
| 1.5.4 BROSTALLICIN..... | 85 |
| 1.5.4.1 In vitro and in vivo brostallicin antitumour activity | 87 |
| 1.5.4.2 Brostallicin Combinations..... | 88 |
| 1.6 PERSPECTIVES ON THE COMBINATIONS OF DEMETHYLATING MOLECULES WITH CYTOTOXIC DRUGS..... | 90 |
| 2. AIMS..... | 92 |
| 3. MATERIALS AND METHODS | 95 |
| 3.1 CELL CULTURE | 96 |
| 3.1.1 Culture Conditions | 96 |
| 3.1.2 Long term storage of cells..... | 97 |
| 3.1.3 Cell transfection and clone selection..... | 97 |
| 3.1.4 Growth inhibition assays..... | 99 |
| 3.1.5 Statistical analysis | 101 |
| 3.1.6 Drug solutions | 101 |
| 3.2 DNA EXTRACTION..... | 102 |
| 3.2.1 Genomic DNA extraction from xenograft tissue samples | 102 |
| 3.2.2 Genomic DNA extraction from human biopsies..... | 102 |
| 3.2.3 Genomic DNA extraction from human blood..... | 103 |

| | |
|---|-----|
| 3.3 METHYLATION ANALYSIS | 104 |
| 3.3.1 Sodium bisulfite modification of DNA..... | 104 |
| 3.3.2 Amplifications of bisulfite modified genomic DNA by Methylation Specific PCR | 106 |
| 3.3.2.1 Assessment of bisulfite DNA modification by amplification of the calponin gene | 106 |
| 3.3.2.2 Methylation Specific PCR of GSTP1 promoter..... | 107 |
| 3.3.2.3 Methylation Specific PCR of GSTM1 promoter | 108 |
| 3.3.3 Pyrosequencing: general description..... | 109 |
| 3.3.3 Pyrosequencing: GSTP1 and MAGE1A promoter methylation analysis | 111 |
| 3.4 <i>IN VIVO</i> EXPERIMENTS | 113 |
| 3.4.1 Animals | 113 |
| 3.4.2 Tumour model..... | 113 |
| 3.4.3 Drug administration | 113 |
| 3.4.4 Evaluation of antitumour activity and toxicity..... | 114 |
| 3.4.5 Statistical analysis | 115 |
| 3.4.6 GSTpi expression | 115 |
| 3.4.6.1 Immunohistochemistry..... | 115 |
| 3.5 WESTERN BLOTTING..... | 116 |
| 3.5.1 Protein extract preparation | 116 |
| 3.5.2 Determination of protein concentration | 117 |
| 3.5.3 SDS-PAGE..... | 117 |
| 3.5.4 Protein transfer and detection | 118 |
| 3.6 Assay principle of total GST Enzymatic Activity | 120 |
| 3.6.1 Total GST activity on cell LNCaP and Du145 cells..... | 120 |
| 3.6.2 KINETIC ASSAYS | 121 |
| 3.6.2.1 Inhibition of the GST activity by brostallicin | 121 |

| | |
|--|-----|
| 3.6.2.2 GST activity with brostallicin | 122 |
| 4. RESULTS | 123 |
| CHAPTER 4.1 | 124 |
| ANTITUMOUR ACTIVITY OF BROSTALLICIN ON HUMAN PROSTATIC CANCER CELLS: <i>IN VITRO</i> COMBINATION WITH DEMETHYLATING AGENTS | 124 |
| 4.1.1 INTRODUCTION | 125 |
| 4.1.2 RESULTS | 126 |
| 4.1.2.1 Differential toxicity of brostallicin on LNCaP and DU145 prostatic cancer cell lines | 126 |
| 4.1.3.2 Evaluation of the activity of the combination of demethylating drugs with brostallicin on LNCaP cell line..... | 128 |
| 4.1.2.3 Effect of combination of zebularine and trichostatin A with brostallicin on LNCaP cells | 133 |
| 4.1.2.4 Effects of the zebularine-brostallicin combination on Du145 and LNCaP- GST cells..... | 133 |
| 4.1.3 DISCUSSION | 137 |
| CHAPTER 4.2 | 139 |
| IN VIVO COMBINATION OF THE DEMETHYLATING DRUG ZEBULARINE AND THE MINOR GROOVE BINDER BROSTALLICIN..... | 139 |
| 4.2.1 INTRODUCTION | 140 |
| 4.2.2 RESULTS | 140 |
| 4.2.2.1 Antitumour activity of zebularine-brostallicin combination in LNCaP xenografts in SCID mice..... | 140 |
| 4.2.2.2 Assessment of re-expression of GSTp in LNCaP xenografts after zebularine treatment..... | 146 |
| 4.2.2.3 Methylation analysis of the GSTpi promoter..... | 149 |

| | |
|---|-----|
| 4.2.3 DISCUSSION | 153 |
| CHAPTER 4.3 | 155 |
| INVOLVEMENT OF GST ENZYMES IN THE ANTITUMOUR ACTIVITY OF BROSTALLICIN | 155 |
| 4.3.1 INTRODUCTION | 156 |
| 4.3.2 RESULTS | 157 |
| 4.3.2.1 Role of different cytosolic classes of GST in the antitumour activity of brostallicin..... | 157 |
| 4.3.2.2 Affinity studies of brostallicin for classes μ and π of GST | 158 |
| 4.3.2.3.GST μ methylation analysis in LNCaP tumours..... | 162 |
| 4.3.3. DISCUSSION | 165 |
| CHAPTER 4.4 | 167 |
| METHYLATION ANALYSIS OF THE MAGE 1A GENE IN A PHASE I STUDY OF DECITABINE IN PATIENTS RECEIVING EPIRUBICIN, CISPLATIN AND 5-FU FOR ADVANCED GASTRIC OR OESOPHAGEAL CANCER | 167 |
| 4.4.1 INTRODUCTION | 168 |
| 4.4.2 RESULTS | 169 |
| 4.4.2.1 Methylation analysis of MAGE 1A gene..... | 169 |
| 4.4.3 DISCUSSION | 172 |
| 5. GENERAL DISCUSSION | 175 |
| 6. REFERENCES..... | 182 |
| 7. APPENDIX..... | 194 |
| 7.1 LIST OF ABBREVIATIONS | 194 |
| 7.2 LIST OF PUBLICATIONS | 198 |

List of figures

| | |
|--|-----|
| Figure 1.3.1 Loss of GSTpi caretaker activity in prostate cells | 41 |
| Figure 1.3.2 Cellular pathways affected by methylation and involved in prostate cancer development. | 44 |
| Figure 1.3.3 Molecular pathogenesis of prostate cancer..... | 45 |
| Figure 1.5.3.1 Kinetic of DNA demethylation induced by 5-aza-dC | 74 |
| Figure 1.5.4 Brostallicin structure and reaction..... | 86 |
| Figure 3.3.1 Sodium bisulfite reaction with cytosine residues | 105 |
| Figure 3.6.1 Reaction of GSH with CDNB catalyzed by GST family of enzymes | 120 |
| Figure 4.1.1 Analyses of LNCaP and Du145 cell lines | 127 |
| Figure 4.1.2 Characterization of LNCaP-GSTpi clones | 129 |
| Figure 4.1.3 Effects of 5-aza-dC on LNCaP cells..... | 131 |
| Figure 4.1.4 Effect of combination of procaine or procainamide and brostallicin on LNCaP cells | 132 |
| Figure 4.1.5 Effects of 96h treatment with zebularine on LNCaP cells | 134 |
| Figure 4.1.6 Effect of the combination of zebularine and TSA on brostallicin toxicity in LNCaP cells | 135 |
| Figure 4.1.7 Effects of combination of zebularine and cDDP or brostallicin on GST expressing cells | 136 |
| Figure 4.2.1 Toxicity of zebularine in untransplanted SCID mice | 141 |
| Figure 4.2.2 <i>In vivo</i> antitumour activity of zebularine-brostallicin combination on LNCaP transplated mice | 143 |
| Figure 4.2.3 Antitumour activity of brostallicin on LNCaP-GSTpi tumours | 144 |
| Figure 4.2.4 Western blotting for GSTpi expression in LNCaP tumour extracts..... | 146 |
| Figure 4.2.5 Morphology and GSTpi expression in LNCaP tumours by IHC..... | 148 |
| Figure 4.2.6 CpG island of the GSTP1 promoter | 150 |

| | |
|--|-----|
| Figure 4.2.7 Agarose gel analysis of MSP of the GSTP1 promoter | 151 |
| Figure 4.2.8 Pyrosequencing of the GSTP1 promoter | 152 |
| Figure 4.3.1 CDNB assay on LNCaP wt and MCF-7 wt cells..... | 157 |
| Figure 4.3.2 Inhibition properties of brostallicin towards GSTpi..... | 159 |
| Figure 4.3.3 Inhibition properties of brostallicin towards GST μ | 160 |
| Figure 4.3.4 Effects of brostallicin concentration on the velocity of reaction with GST enzymes..... | 161 |
| Figure 4.3.5 CpG island of GSTM1 promoter | 163 |
| Figure 4.3.6 MSP on bisulfite modified GSTM1 promoter..... | 164 |
| Figure 4.4.2 Methylation analysis of blood samples by PSQ | 171 |
| Figure 4.4.3 Methylation analysis of tumour samples by PSQ..... | 173 |

List of Tables

| | |
|---|-----|
| Table 3.1.1 Drug solutions..... | 101 |
| Table 3.3.1 MSP conditions for GSTM1 | 108 |
| Table 4.2.1 Parameters related to the <i>in vivo</i> treatments of the zebularine-brostallicin combination on LNCaP tumours..... | 142 |
| Table 4.2.2 LNCaP-GST tumour sensitivity to brostallicin..... | 145 |
| Table 4.2.3 Summary of IHC detection of GSTpi in LNCaP tumours | 147 |
| Table 4.3.1 Cytotoxicity of brostallicin in MCF-7 clones | 158 |
| Table 4.2.1. Parameters related to the <i>in vivo</i> treatments of the zebularine-brostallicin combination on LNCaP tumours..... | 142 |
| Table 4.2.2 LNCaP-GST tumour sensitivity to brostallicin..... | 145 |
| Table 4.2.3 Summary of IHC detection of GSTpi in LNCaP tumours | 147 |
| Table 4.3.1 Cytotoxicity of brostallicin on MCF-7 clones | 158 |

1. INTRODUCTION

1.1 THE EPIGENETIC CODE

The primary sequence of the four nucleotides in DNA represents the first layer of the information stored in the nuclei of cells. These 2-3 billion bases of DNA, encoding about 30000 genes, form, with the histone proteins, the nucleosomes. In these uniformly distributed units, a 146bp stretch of DNA is wrapped around a core particle of two heterodimers, H2A-H2B and H3-H4 (Khorasanizadeh, 2004). Transcription and translation are the means by which cells read the genetic instructions in the DNA sequence. These processes are strictly regulated through the binding of sequence-specific DNA binding proteins to regulatory promoters and enhancers. Recently, it has been discovered that non-coding RNA molecules can recognize complementary sequences of DNA or RNA in order to transcriptionally inactivate them, before that other DNA modifications occur: in fact it seems that once RNAi binds DNA, this is set to stop transmission of genetic information. This is a common event in *Saccharomices pombe* and in *Drosophila*, but also in plants and mammals (Bird, 2002).

Superimposed upon this “primary structure” is a layer of heritable modifications of the chromatin components that we have only just begun to analyse and appreciate. This epigenetic information is mainly stored as chemical modifications to cytosine bases and to the proteins that package the genome (Lande-Diner and Cedar, 2005; Berger, 2007).

By regulating chromatin structure and DNA accessibility, these chemical changes influence how the genome is made manifest across the diverse array of developmental stages, tissue types and disease states. However one critical function of chromatin modifications is to distinguish euchromatin, where DNA is kept accessible for transcription, and heterochromatin, where chromatin is “inaccessible”; but unlike we thought few years ago, this is not “junk” DNA.

Heterochromatin has a fundamental role in the maintenance of functional chromosome structure such as centromeres and telomeres, thus allowing the proper sister chromatid

cohesion and chromosome segregation; it also stabilizes repetitive DNA sequences by inhibiting recombination between homologous repeats.

A DNA modification can be defined as “**epigenetic**” if it does not change DNA sequence and it is, at the same time, heritable through subsequent cell generations. The heritability is a fundamental aspect of epigenetics (besides its effects on gene expression), which allows us to not confuse it with transcriptional regulation, which are transient changes occurring during a cell life (Bird, 2002).

Epigenetic modifications are not a common event in unicellular organisms, but they involve the animal genome in any particular cell type: this phenomenon depends on different mechanisms, working independently or together to ensure the maintenance of the transcriptionally competent chromatin or its proper structure through cell generations. In many species, these non-genetic alterations of the DNA sequence are represented by covalent modifications of histone proteins and by DNA methylation (Li, 2002).

1.1.2 DNA methylation

DNA methylation is a common event in eukaryotes, especially in mammals, but it does not occur in yeast or in *C.elegans*.

In general, among the four DNA bases, C and A can be further modified by a post-replicative chemical addition of a methyl group, by specific DNA methyltransferases (DNMTs), which use S-adenosylmethionine as the methyl group donor.

⁶N-methyladenine is usually found in plants, and it is a very specific DNA modification in their mitochondrion genome, where there is no cytosine methylation.

Up to now we have no strong insights about the role of adenine methylation in higher eukaryotes. Certainly, plants are characterized by a more differentiated set of DNA methyltransferases, and this could justify the experimental data showing that knock out of the plant enzyme homologous to animal *dnmt1* is not lethal for plants.

In bacteria, methylation has both a regulatory and a protective role; in fact, restriction-modification systems contain a DNA methylase that protects self DNA sequences from digestion with its cognate restriction endonuclease which, conversely, digest unmodified foreign DNA, such as viral DNA (Vanyushin, 2005).

Cytosines targeted to be methylated by DNMTs belong to a CG dinucleotide. This dinucleotide is generally underrepresented in the genome, because ^mC can be a mutagenic modification, due to the spontaneous oxidative deamination of ^mC to T. In fact, the human genome shows the strongest suppression of CpG, thus resulting to be the most infrequent dinucleotide in the genome. Methylation occurs globally in the genome, but not in CpG “islands” (Bernstein et al., 2007), which are DNA regions of >500bp, having a high CpG density (CpG observed/CpG expected ratio >0.6) (Gardiner-Garden and Frommer, 1987; Takai and Jones, 2002), usually localized upstream of about half of mammalian genes. Those CpG dinucleotides residing outside the CpG islands are usually localized within repetitive elements, where they are heavily methylated in somatic tissues.

Methylation was already known for its role in setting up the chromatin structure in imprinted regions or the X chromosome (Jaenisch and Bird, 2003).

The first insights linking methylation and gene silencing come from analysis of pericentromeric heterochromatin. Transcriptionally competent regions of DNA, which constitute the euchromatin of the genome, show a more variegated picture as regard methylation: that's why they must ensure that the proper level of gene expression can be stably maintained in the daughter cells in a heritable way.

Promoters can be differentiated regarding their CpG content: those containing CpG islands (about 50%) usually are methylation free and the nucleosomes are associated with highly acetylated histones.

In these promoters, methylation does not normally participate in regulating the transcription of downstream genes, whether such genes are being actively transcribed or not. However it is necessary to emphasize that methylation is not a transcriptional

regulatory mechanism, and these promoters are kept free of methylation, irrespective of gene expression levels; this usually depends on the associations of “negative” or “positive” factors.

The role of DNA methylation in CpG-poor promoters is not clear; so it is not known for how many of them CpG methylation might have a modulator role in their transcription. Some studies show that individual CpG sites in such promoters can determine the transcriptional status of a gene by blocking the access of certain transcription factors that are sensitive to the methylation of CpG in their sequence. Alternatively, the methylation of sites in these regions might also silence genes by helping to recruit protein complexes that repress transcription (Jones and Baylin, 2002).

In both cases described above, we cannot forget that mechanisms involving histone modification are as important as DNA methylation in setting up the proper conformation of chromatin (Berger, 2007; Kouzarides, 2007).

In proliferating primordial germ cells there is a general erasure of methylation, also involving the parental imprinted regions and the X chromosome in females. Once the parental imprints have been erased, new imprints must be re-established according to the gender. This re-establishment occurs only after gender-determination has been initiated and male and female germ cell development diverges to give rise to sperm or oocytes, respectively (Sasaki and Matsui, 2008). Methylation time is different in males and in females, because it occurs before meiosis in males, in prenatal prospermatogonia (diploid stage), whereas methylation is acquired postmeiotically in the female germ line, in particular in growing oocytes that are arrested at the diplotene stage of meiosis I. The early methylation trigger in males accounts for deeper effects that this modification has on the entire male genome. In fact, because cytosine methylation results in an increased C→T mutation rate (Momparker and Bovenzi, 2000) and spermatogonia maintain methylation patterns for a large and variable number of mitotic divisions before entering into meiosis, the mutational pressure has led to a low density of CpG dinucleotides in paternally

methylation differentially methylated regions (DMRs) relative to maternal DMRs. Errors in the establishment or maintenance of germ cell methylation patterns can cause human diseases (Schaefer et al., 2007).

Moreover, DNA methylation patterns are physiologically set during the first steps of development, when they are erased in the morula and established de novo during implantation. Target sequences for de novo methylation are transposons, clustered repeats (primarily, pericentric satellite DNA) and, to a lesser extent, single-copy gene sequences (Reik, 2007).

Single copy genes and their promoters are usually protected from methylation, but they can become aberrantly modified in the developing early stages of human cancers, thus causing the silencing of genes, such as tumour suppressor.

That all chromatin is not compacted into a “closed” form (an event only occurring in the mammalian female X chromosome) and that euchromatin and transcriptional active regions are not involved in silencing, is related to the presence of some specialized DNA elements known as boundary elements, which apparently serve as barriers against the propagation of the inactive form of chromatin (Grewal and Moazed, 2003).

In summary, methylation patterns are set in two different periods in the mammal cells: germ cell development and early embryogenesis, in both cases after its erasure in the total genome (Li, 2002). This ensures that transcriptionally silenced regions can be stabilized and transmitted to the next generations. Thus, from a physiological point of view, methylation seems not to be a simple regulatory mechanism of the genome, but rather a system of cellular memory, which propagates the silent state.

1.1.3 DNA methyltransferases and Methyl Binding Proteins

DNA methylation is mediated by the enzymatic activity of a family of proteins, named DNA methyltransferases (DNMTs). At the moment, DNMTs from 1 to 3b have been described (Goll and Bestor, 2005).

Typically, DNMT1 is responsible to complete DNA methylation during replication, when the new synthesized “daughter” DNA strand needs to be methylated using the parent strand as template. In this way, methylation is maintained through subsequent generations. This role justifies the name of “maintenance” methyltransferase for DNMT1; it exerts its function during the S phase of the cell cycle and, for this reason, it moves from a diffuse nucleoplasmic distribution to sites of DNA synthesis (replication foci). DNMT1 ensures that methylation of both DNA strands is maintained during replication by a semi-conservative mechanism. DNMT1 expresses at least two splice variants, named DNMT_o and DNMT_p: DNMT_o is specific to oocytes and to preimplantation embryos, while DNMT_p has been found in pachytene spermatocytes (Robertson, 2002).

DNMT2 does not show any methyl-transfer activity, even though it is the most highly conserved among all DNMTs across species; in fact DNMT2 homologues have been found in plants, yeast, flies, mice and humans (Dong et al., 2001). Human DNMT2 protein is primarily localized to the cytoplasm, in contrast to the exclusively nuclear localization of DNMT1 and DNMT3. This is because the DNMT2 target is a C in the anticodon loop of tRNA^{Asp}, which is strictly conserved in all genomes encoding DNMT2. The exact role of this modification is still unknown (Goll et al., 2006).

DNMT3s are generally referred as de novo methyltransferases, because they are responsible for initiating the methylation pattern in the genome, both at the germ cell level and in the embryo. Different enzymes belong to this family. De novo methylation is established by DNMT3a/b enzymes, which generally reside in pericentromeric heterochromatin (Weisenberger et al., 2004).

DNMT3a activity is not strictly associated with a specific phase of the cell cycle and can be found at discrete nuclear foci throughout the cell cycle; it is required in male germ cells for the establishment of methylation imprints at DMRs and to ensure the proper methylation pattern on transposons and pericentric repeats.

DNMT3b appears to be specialized for the establishment and/or maintenance of DNA methylation of the minor satellite repeats (satellites 2 and 3 on human chromosomes 1, 9 and 16). It has several isoforms, resulting from alternative splicing, and their expression is tissue-specific; among them, DNMT3b4 and DNMT3b5 are expressed predominantly in the testis (Robertson, 2002).

DNMT3L is unique, because it regulates the DNMT3A and 3B function in their establishing methylation in prospermatogonia (Li, 2002; Sasaki and Matsui, 2008).

Further insights into DNMTs functions come from experiments involving KO mice (Goll and Bestor, 2005; Robertson, 2002). Thus the *Dnmt1*^{-/-} genotype is lethal and knock out embryos failed to develop beyond mid-gestation. KO fibroblasts are characterized by a severe demethylation, also affecting imprinted regions. ES cells lacking *Dnmt1* grow normally in the undifferentiated state, but die by apoptosis when induced to differentiate *in vitro* and *in vivo*.

The fact that *Dnmt2* is not involved in maintenance or in setting methylation is underlined by the normal phenotype of *Dnmt2*^{-/-} murine ES cells, thus suggesting that this enzyme is required for neither maintenance nor de novo methylation.

The *Dnmt3a*^{-/-} mouse phenotype appears to be normal at birth, but by 4 weeks of age, all the mice die. In contrast, *Dnmt3b*^{-/-} mice die at birth. *Dnmt3a* and *3b*^{-/-} ES cells exhibit comparable degrees of demethylation, although for *Dnmt3b*^{-/-} cells, a decrease in methylation level at minor satellite repeats in the pericentromeric region has been reported. The double *Dnmt3a*^{-/-}*Dnmt3b*^{-/-} genotype is lethal for mouse embryos and ES double mutant cells show a dramatic reduction of the global level of methylation compared to the single *Dnmt3* KO.

DNMTs are able to bind many proteins with different functions: among them, histone deacetylases (HDAC) and methyl binding proteins (MBDs) are also involved in epigenetic modifications.

MBD proteins read the methylation “language” on DNA, because they specifically bind the methylated CpGs. A family of five methyl-CpG-binding proteins has been characterized, and each of them contains a region closely related to the methyl-CpG-binding domain (MBD) of MeCP2 protein, the founding member of this family, whose important role in maintaining gene silencing through the binding to ^mC is underlined by a severe neurological disease, the Rett syndrome, which specifically depends on MeCP2 gene mutations. In addition to MeCP2, MBD1, MBD2 and MBD3 have been implicated in methylation-dependent repression of transcription. MBP1 seems to exert its function during S phase of the cell cycle and it is also able to interact with the histone3lysine9 (H3K9) methyltransferase enzyme, SETDB1, so coupling recognition of DNA methylation to modification of the surrounding chromatin by histone methylation (Klose and Bird, 2006).

1.1.4 Histone Code

There is cross-talk between signals modifying DNA and those modifying histones, and the result is the transcriptional state of the genome. While DNA methylation has a repressive meaning for transcription, histone proteins can be modified in a more variegated way, both for the kinds of modifications and for their consequences, i.e. activating or repressive. All modifications occurring on the histones set up the so-called “histone code” (Kouzarides, 2007).

Chemical modifications targeting histones occur especially at N-terminal unstructured tails, which extend out from the globular regions, that are associated with DNA in nucleosomes (Berger, 2007). This regulatory mechanism is strongly conserved, because is

common to very different eukaryotes, from *Drosophila* to yeast and mammals (Khorasanizadeh, 2004).

The covalent modifications that take place on histones include the acetylation, ubiquitination and sumolation of lysines, mono-, di-, tri-methylation of lysines and arginines, the phosphorylation of serines and threonines, and the ADP-ribosylation of glutamic acids. All of these modifications, except for methylation, appear to be reversible. There has been much effort in the last few years to identify the enzymes responsible for these modifications. At the moment better characterized are the methyltransferases and kinases, which are also targeted by many signalling pathways. These modifications affect the contacts between different histones in adjacent nucleosomes or the interaction of histones with DNA, generally by the alteration in the histone charge, which in turn has structural consequences for the chromatin architecture. For example, acetylation has the strongest potential to unfold chromatin, since it neutralizes the basic positive charges of the lysines, and hence the ionic bond to the negatively charged phosphate groups in DNA. Many different kinds of modifications occur on lysine residues and these need a strong regulation of the enzymes involved in setting them. Generally, heterochromatic domains are associated with low levels of histone lysine acetylation and high levels of certain lysine methylation sites, typically H3K9, H3K27, H4K20, while euchromatin has high levels of acetylation and is trimethylated at H3K4, H3K36 and H3K79.

The functional consequences of histone post-translational modifications (PTMs) can be direct, causing structural changes to chromatin, or indirect, acting through the recruitment of effector proteins. For example, H3K4^{met} recruits protein complexes containing bromodomains, which are responsible for acetylation; moreover H3K4^{met} effects could also depend on different external circumstances (for example, physiological or damaged state) or on a different step (active transcription or a silenced state) of the cell cycle. Surely, different meanings associated to the same modification could be more flexible than only a kind of information during activities occurring at dynamic transcribed regions.

It is important to note that many kinds of modifications could not be simply classified as activating or repressing, but they have the potential to activate or repress gene expression under different conditions.

It is matter of debate whether histone modifications are epigenetic or not, because many of them are removable; undoubtedly they have been implicated in a number of epigenetic phenomena. I have already pointed out that a chemical modification of chromatin is epigenetic only if heritable through subsequent generations: at the moment such a mechanism has been proposed only for H3K9 in the transmission of the heterochromatin (Khorasanizadeh, 2004).

Together DNA methylation and histone modifications represent a second layer of the information carried by the genome, which is not encoded by the nucleotide sequence.

1.1.5 Nucleosome Remodeling

ATP-dependent chromatin remodelling complexes, called SWI/SNF2 represent an upper level of chromatin modifications: they operate at the nucleosome level by using the energy of ATP-hydrolysis to introduce superhelical torsion into nucleosomal DNA with the main consequence to expose DNA bulges or loops, which modify the accessibility of chromatin to different proteins controlling transcription, DNA replication, recombination and other biological processes. The fundamental role of this function is underlined by the fact that ATPase is the most conserved subunit, among numerous others, which at the moment are not well characterized (Li, 2002).

The chromatin remodelling complexes are involved in at least three types of activities: octamer sliding, DNA looping and histone substitution, in general occurring when, following replication, the bulk of new synthesized histones needs to be deposited on the nucleosome.

To conclude, epigenetic modifications set up a pattern of different level of expression in specific cellular phenomena, including tissue-specific gene expression, cell differentiation,

genome imprinting, X chromosome inactivation, regulation of chromatin structure, aging; their deregulation are involved in many diseases, above all cancer (Jaenisch and Bird, 2003).

1.2 EPIGENETICS AND HUMAN DISEASES

In the last decade much effort has been focused on understanding the heritable causes of human diseases besides genetic mutations or chromosomal aberrations.

The potential disease determinant of epigenetics depends on its heritability during cell division in a given cell lineage; another important factor characterizing epigenetic regulatory patterns is its “sensitivity” to environmental factors, acting over time (Bjornsson et al., 2004; Feinberg, 2007). As a consequence, epigenetic modifications of DNA have to be heritable and stable through cell generations, but flexible enough to undergo changes when necessary, mainly during cellular differentiation and in response to modified environmental stimuli.

It is questionable whether the concept of heritability could be applied only to subsequent cell generations in only an organism or also to transmission of characters from parents to the offspring, because, as I mentioned in the previous part of this introduction, during early embryogenesis, there is an extensive erasure of the parental pattern of genome methylation, except for imprinted regions.

1.2.1 Imprinting disorders

Imprinting disorders derive from specific defects involving those chromosome regions differentially expressed between paternal and maternal alleles. The control of gene expression within imprinted domains is exerted by imprinting control regions (ICR), often acting over large distances: differential methylation in ICRs is generally established in germ cells and maintained throughout development. Loss of imprinting (LOI) is the disruption of imprinted epigenetic marks through gain or loss of DNA methylation, or simply the loss of normal allele-specific gene expression.

The aberrant imprinted loci, identified up to now, are located on chromosomes 11, 15, 20 and are responsible for many different disorders.

The Beckwith-Wiedemann syndrome (BWS) comprises deregulation on two neighbouring subdomains on 11p15: one is H19/IGF2 (imprinted, maternally expressed, untranslated mRNA/ insulin-like growth factor (IGF) 2), which is regulated by a differentially methylated region (DMR), methylated on the paternal, but not on the maternal allele. Some BWS patients show loss of imprinting of IGF2, which leads to a double dose of this autocrine factor, resulting in tissue overgrowth and in an increased cancer risk. The other subdomain is regulated by another DMR, which is methylated on the maternal but not on the paternal allele; among the genes in this deregulated region is p57kip2, a cyclin-dependent kinase inhibitor.

The Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS) affect the same imprinted locus at 15q11-q13 that contains both paternally and maternally expressed genes. While PWS arises from loss of the paternally expressed genes, the loss of maternally expressed genes determines AS.

Another pair of human disorders caused by opposite alterations at the same locus is Albright hereditary osteodystrophy (AHO) and pseudohypoparathyroidism (PHP1A). These diseases are related, because both arise from the deregulation of the same imprinted locus on chromosome 20q13, called GNAS, which encodes the α subunit ($Gs\alpha$) of the heterotrimeric GTP-binding protein Gs. The two disorders are differentially inherited: while AHO is paternally transmitted, PHP1a shows a maternal transmission. Both diseases display mental retardation and subcutaneous ossification, but PHA1A patients also show resistance to the peripheral action of several hormones involved in activating pathways coupled to Gs.

It is important to note that the bulk of CpG dinucleotides, outside the CpG islands, resides in the intergenic and intronic regions of DNA, particularly within repeat sequences and transposable elements: these cytosines carry a strong methylation (about 70-90% of CpG dinucleotides are methylated), which ensures the proper structure to heterochromatin and silencing of repeat sequences and transposable elements. Chromosome instability could

result from expansion of trinucleotide repeats during gametogenesis. Depending on the nucleotide sequence in the repeats, these triplets can be methylable, as in the case of CGG repeats, or not: in the first case, an increased level of methylation results in an aberrant silencing and leads, among the other possible diseases, to the Fragile X Syndrome (FRAXA). This X-linked disorder causes mental retardation in about 1 in 4000 males. It is characterized by an extensive increase of the CGG repeats (from 6-52 copies in a normal situation to more than 200 copies in the pathological status) within the 5'-untranslated region of FMR1 gene which encodes an RNA-binding protein expressed in many foetal and adult tissues. In contrast to hypermethylation due to increased numbers of CGG triplets, other epigenetic disorders come from the contraction of repeats, thus causing hypomethylation: An example is represented by the Facioscapulo-humeral muscular dystrophy, with the loss of copies of the polymorphic 3.3kb D4Z4 repeats (Robertson, 2005).

In summary, diseases could depend either on DNA hypermethylation, which leads to an aberrant silencing, or hypomethylation, which could make the affected loci more fragile and unstable or allow an aberrant overexpression of specific genes.

Other disorders arise from defects in the molecular machinery regulating the setting or the control of methylation. For example, in systemic lupus erythematosus (SLE), reduced levels of DNMT1 result in a global hypomethylation of T cell genomes (Richardson, 2003). Similarly, a heterozygous mutation in the DNMT3b gene is associated with the decondensation of pericentromeric heterochromatin on chromosomes 1 and 16 in ICF (Immundeficiency, Centromeric region instability and Facial anomalies) patients.

However, in addition to intrinsic cell factors whose defects lead to a deregulated overall/gene-specific methylation, extrinsic factors, such as environmental toxins like heavy metals, can disrupt cellular DNA methylation patterns in the cells (Hatchwell and Greally, 2007; Feinberg, 2007).

Recently great interest has been generated by the consequences of assisted reproductive technologies (ART) on the epigenetic programming of the embryo; in fact it has been demonstrated that children born through ART have an increased frequency of developing BWS and AS (Wilson et al., 2007; Robertson, 2005).

1.2.2 Epigenetics and Cancer

The first sign of an epigenetic origin of neoplasia derived from abnormalities in gene imprinting found in cancer. In particular a double dose of the IGF2 growth factor has been found in apparently normal colonic epithelium of patients with colorectal cancer and this appears to result from loss of imprinting of the region regulating IGF2 expression. The relationship between double doses of IGF2 and cancer risk was further supported by data from mice engineered for Igf2 biallelic expression, which show a higher proportion of undifferentiated cells (Feinberg and Tycko, 2004).

Gene silencing by methylation fits well with Knudson's two hit model (Knudson, 2000): when one of two alleles is mutated in the germ line of a patient with a familial form of cancer, and the resultant tumour retains both alleles of the gene, hypermethylation is commonly seen as the second inactivating change (Herman and Baylin, 2003).

While the earlier efforts in cancer epigenetics were focused on understanding the involvement of changes in DNA methylation with respect to both aberrant hypermethylation of CpG in gene promoters and hypomethylation, it is now clear that abnormalities comprising a multitude of aberrations in virtually every component of chromatin can be involved in carcinogenesis. The mechanism of epigenetic heredity seems to share the same characteristics with genetic alterations, mainly because they are transmitted faithfully during mitosis, thus undergoing the same selective process in the development of cancer and collaborating together in its evolution. Nevertheless, epigenetic events can potentially occur at an increased rate compared to mutations in somatic cells.

DNA methylation patterns are profoundly modified in tumours. Not only are there increased levels of methylation in the promoters of genes involved in carcinogenesis, but it is now emerging that alterations are genome-wide. Also we have to think of a general change in 5-methylcytosine distribution rather than an overall increase in the total amount of methylation (Jones and Baylin, 2007).

However there is an emerging idea that some tumours can be characterized by the concordant methylation of a specific set of genes, arising from the so-called “CpG island methylator phenotype” (CIMP) (Issa, 2004).

The first epigenetic abnormality discovered in tumours was hypomethylation, occurring both in heterochromatin and euchromatin. In the latter case, the main consequence is the activation of genes conferring a growth advantage to tumours. For example, hypomethylation of the MDR1 promoter was linked to its overexpression and acquirement of multidrug resistance in acute myelogenous leukaemia. Alternatively, if hypomethylation occurs at pericentromeric satellite sequences, it might predispose to their breakage, aberrant recombination and, in general, to genomic instability (Gaudet et al., 2003), that is a hallmark of cancer (Hanahan and Weinberg, 2000).

Although hypomethylation was the first epigenetic modification discovered, researchers' attention was initially concentrated on hypermethylation of CpG islands in the promoter region of genes involved in cancer development. The list of genes that are inactivated by hypermethylation is becoming more and more extensive and involves genes belonging to different pathways and responsible for the onset of different kind of tumours.

Like for genetic changes, researchers analyzed the timing of epigenetic changes and it was concluded that these play a seminal role in the earliest steps of cancer initiation and the so called “epigenetic gatekeepers” were identified. For example, the tumour suppressor p16 is one of the most common and earliest epigenetically mediated losses in human cancer. Its silencing occurs in a subset of preinvasive stages of breast, colon, lung and other cancers.

Undoubtedly, whatever is the modification, i.e. genetic or epigenetic, it must push the early aberrant clonal expansion of cells (Feinberg et al., 2006).

If both genetic and epigenetic modifications can result in aberrant gene function, thus allowing the survival and clonal expansion of transformed cells, how do they differ? The first answer could be that gene mutations in a single tumour are seldom multiple in a given pathway, since selection for one hit appears sufficient to produce full pathway disruption, while multiple epigenetic events may frequently affect a single-cell pathway. If these events occur in a cancer stem cell, together with the deregulation of the polycomb complex of protein (PcG) system and histone modifications, they provide an advantage for cell growth and hence tumour expansion. There is not always a direct cause and effect role in gene silencing, but it is becoming clear that whole groups of genes may be inactivated as a part of an abnormal program (Jones and Baylin, 2007).

There is an emerging relationship between the already known mutations, including chromosomal aberrations that occur in tumours, and epigenetic modifications, above all because studies performed until now have highlighted that the latter are not sporadic events, but alterations affecting more or less each pathway associated with tumour initiation and progression in every kind of tumour. By this mechanism of silencing, the expression of tumour suppressor genes in the cells can be reduced or eliminated and it constitutes an alternative mechanism for transformation to gene mutations. This might imply that genes that are inactivated by mutations are not, at the same time, the target for methylation. However aberrant epigenetic modification can progressively affect gene expression as result of the prolonged exposure to dangerous molecules, such as carcinogens, or of a particular diet (for example, dietary supplements with folate or vitamins can exert marked effects on the incidence of colon cancer) (Jaenisch and Bird, 2003).

Genome wide methylation analysis allowed researchers to identify genes that are silenced in different stages of tumour progression or in a specific kind of tumour, thus giving a

biomarker for tumour type, or for risk assessment or indicators of susceptibility or response to therapy. But researchers' interest has also been focused on the assessment of the role of epigenetics in malignant transformation. In fact, the idea of a seminal role of epigenetics in acquiring an aberrant phenotype, which is then followed by genetic changes and clonal expansion, has begun to be generally accepted (Feinberg et al., 2006).

Our view of cancer progression is often anchored to the sequential genetic changes described by Vogelstein (Kinzler and Vogelstein, 1996) for colorectal tumourigenesis and characterized by a continued clonal selection that gives rise to tumour proliferation, invasion, metastasis and drug resistance. In this model of cancer onset, epigenetic changes are viewed as surrogate alterations for genetic changes, by simply mimicking their effects on gene expression.

From the epigenetic point of view, the tumour genome is characterized by an extensive and general hypomethylation and by the hypermethylation of gene promoters (mainly tumour suppressors), which can be tumour-specific (Clark and Melki, 2002). The former arises first and is strongly linked to chromosomal instability and loss of imprinting, while the latter is restricted to promoters.

Increasing numbers of genes are normally found to be methylated at promoters, but hypomethylated and activated in the corresponding tumours, as in the case of melanoma antigen family A, 1 (MAGE1) in melanoma (Ohman and Nordqvist, 2001).

In the last few years, studies on epigenetic alterations have highlighted that they are very early events in tumour aetiology initially affecting pre-cancerous tissues. So it has been proposed that the first step in cancer development could involve the epigenetic disruption of stem/progenitor cells, which might perturb the normal balance between undifferentiated progenitor cells and differentiated committed cells within a given compartment.

Epigenetic deregulation affects the tumour progenitor genes, which regulate "stemness" itself, mainly its pluripotency and unlimited self-renewal and define the balance towards stem cell renewal and proliferation over normal differentiation within a given tissue. The

disruption of tumour-progenitor genes could be a consequence of the microenvironment, and this influence is a mark of the plasticity of the “epigenetics” on DNA.

The first modifications does not alter the normal phenotype of the cells in which they occur; the tumour transformation depends on a second hit, generally an initiating mutation (but also an epigenetic activation of an oncogene or silencing of a tumour suppressor) within the subpopulation of epigenetically disrupted progenitor cells: this mutation is specific for the tumour type.

The transformation process itself comes to an end when cells become able to evolve their transformed phenotype, through further genetic or epigenetic events, such as telomere erosion, chromosomal rearrangements etc.

The epigenetic progenitor model of cancer mainly explains phenomena such as environmental influence and the common occurrence of late metastatic disease. It also strongly supports the use of epigenetic therapies, which exploit the reversibility of epigenetic modifications.

Examples of early epigenetic modifications include the loss of p16 expression in pre-invasive lung and breast tumours or GSTP1 in the first stages of prostate cancer. These early epigenetic alterations predispose cells to the genetic abnormalities that advance the neoplastic process, but they also act over the entire period of tumourigenesis.

Colon cancer offers a model for tumour progression by epigenetic mechanisms and to understand how both genetic changes and epigenetic gene silencing combine to determine the phenotype of a tumour cell. It has been demonstrated that both alleles of the same gene can be silenced by these two different mechanisms, but that they do not ever occur simultaneously on the same allele (Baylin and Ohm, 2006). The deregulation of Wnt pathway (and its antagonist SFRPs) is one of the mechanisms characterizing the progression from normal epithelial cells to invasive cancer; epigenetic silencing of SFRPs determines a constitutive expression of Wnt, which, in turn, inactivates the well known tumour suppressor adenomatosis polyposis coli (APC) in colon tissues.

Wnt constitutive activation characterizes the early stages, pre-invasive colon lesions, even before the appearance of mutations in the pathway. The fundamental role of SFRPs in determining clonal expansion is demonstrated by experimental data in which deletions of genes that encode DNMTs lead to SFRP promoter demethylation and expression. This results in the downregulation of Wnt signalling and induction of apoptosis, despite the presence of mutations in downstream genes of the pathway (Baylin and Ohm, 2006).

However, the analysis of colorectal cancer has allowed not only the sequence of epigenetic and genetic modifications in carcinogenesis to be defined, but also to appreciate the mechanistic differences between them, using a genome wide approach. Results published by Frigola et al. (Frigola et al., 2006) demonstrated for the first time that epigenetic gene silencing in cancer can encompass a large chromosomal region, and not only a specific CpG island. In fact, samples from patients and colon cancer cell lines (namely HCT 116 and SW 48) demonstrate a contiguous hypermethylation of an entire 4Mb band of chromosome 2q.14.2, containing both regions corresponding to CpG island criteria (as defined by Gardiner and Frommer, 1987) and regions which do not meet the standard definition of a CpG islands. All genes in this region show a significant re-expression after treatment with the demethylating agent 5-aza-deoxycytidine (5-aza-dC) and the deacetylase inhibitor trichostatin A (TSA), whether or not they have a CpG island in the promoter.

Long range epigenetic silencing (LRES) through methylation seems to be specific for cancer cells; in normal cells, X inactivation and imprinting, which also involve a long range silencing, mainly depend on non coding RNA or microRNA transcripts (Baylin and Ohm, 2006).

Another example of the relationship between epigenetic and genetic gatekeeper steps in cancer progression comes from the Hypermethylated In Cancer (HIC1) and p53 genes. HIC1 is a target of the active p53 and becomes hypermethylated in cancer from early, pre-invasive stage breast and colon tumours. Hic1^{+/-} Trp53^{+/-} mice develop osteosarcomas,

breast and ovarian tumours and show deletion of the wild type allele of Trp53. During the early stages of tumour progression, loss of Hic1 expression through methylation results in partial loss of p53 function. Cells that acquire more powerful inactivating mutations in p53 are selected for and contribute to rapid tumour growth and progression (Baylin and Ohm, 2006).

I have generally referred to epigenetic modifications without offering more insights in the kind of modifications that affect the early stages of tumour development. Firstly it seems that histone methylation, mainly H3K9, occurs during the initial phases of gene silencing in cancer and that DNA methylation later spreads over the promoter to “lock in” the transcriptional activity. Actually we know more about the maintenance than the initiation of tumour-suppressor gene methylation (Bachman et al., 2003; Espada et al., 2004).

The fact that epigenetic modifications occur in the early stages of tumour development undoubtedly represents a real potential for drug targeting, as silenced genes can be induced to express again.

I have previously described the molecular machinery supporting the epigenetic modification of chromatin. It is also important to understand if there is a deregulation of this machinery during cancer initiation and progression. Undoubtedly the picture is quite complicated: cancer is characterized by both hypomethylation and hypermethylation of the genome even though, in recent years, many studies have mainly focused on hypermethylation analysis of the promoters of cancer-related genes. However alterations in DNMTs activity and its role in the modification of methylation pattern in cancer are not well understood. Elevated levels and activities of DNMT proteins occur in various cancer types, including gastric, bladder, leukaemia, colon and lung. However the clearly distinct roles of maintenance methylation for DNMT1 and de novo methylation for DNMT3a and 3b do not seem to be strictly respected in cancer. In fact, both genetic depletion and RNA interference against DNMT1 in human colon and other cancer cells resulted only in lower decrease in overall DNA methylation (Ting et al., 2004), minimal loss of promoter

hypermethylation and undetectable re-expression of silenced tumour suppressor genes. But these data are not supported by other studies performed on different cancer models, so the conclusion could be that the threshold requirements for DNMT1 differ in different cancers (Robert et al., 2003; Ting et al., 2006). No significant changes were also detected in DNMT3b ^{-/-} clones, which contain about 97% of wild type genomic 5-methylcytosine content.

Instead extensive demethylation was observed in double knock out (KO) cells for DNMT1 and DNMT3b (corresponding to a roughly 95% reduction in mC content): the lack of these two enzymes affects the methylation level of each kind of “methylatable” target: satellite2 sequences, Alu repeats, but also single copy genes and the imprinted gene Insulin Growth Factor (IGF) 2. In particular, single copy genes have an expression level comparable to the one we can obtain with 5-aza-dC treatment (see later).

The complete erasure of methylation in the double KOs compared to the weak decrease in the single DNMT KO supports the idea that DNMT1 and DNMT3b cooperatively maintain all methylation in the HT116 cell line, both at repeat sequences and at single copy genes. The resulting idea is that there is, at least in part, a functional redundancy among the enzymes responsible for genome methylation and that we cannot clearly distinguish a maintenance DNMT from *de novo* DNMT (Rhee et al., 2002; Leu et al., 2003).

There is little doubt that the complete re-expression of tumour suppressor genes in double KO cells clearly demonstrates that methylation is a silencing mechanism involved in tumourigenesis.

It is well established that tumours have a decreased level of the global 5-methylcytosine level, about 20-60% less than their normal counterpart, that mainly affects the body of the genes involved (the coding regions and introns) and the repetitive DNA sequences, which account for 20-30% of the human genome. The mechanism responsible for genome hypomethylation in cancer is unclear. Several possibilities have been proposed, including insufficient dietary folate or genetic lesions in the folate metabolic pathway, since the liver

of rats, fed with folate deficient diets, exhibits genome hypomethylation and also increased DNA strand breaks and typically develops cancer.

In addition, it is also possible that hypomethylation is a result of the deregulation of the putative demethylase enzymes or conversely due to dysfunctions of the DNA methyltransferases enzymes.

In contrast to the global hypomethylation, gene promoters containing the so-called CpG islands can be hypermethylated and this modification is correlated with transcriptional silencing. Although the most significant proportion of any CpG islands is located in the 5'untranslated region and the first exons of the genes, certain CpG islands can be occasionally be found within the body of the gene, or even in the 3'region: CpG islands in these atypical locations are more prone to methylation. Exceptionally, certain small genes can be considered in their totality as a whole CpG island.

CpG islands (not all, but those ensuring an advantage to tumour progression) will become progressively hypermethylated and this process could be age-related or influenced by environmental stimuli. As I have previously described, DNA methylation occurs by covalent addition of a methyl-group at the carbon 5 of the cytosine ring, resulting in 5-methylcytosine; but the methyl-groups cause gene silencing and a closed chromatin conformation only if they project into the major groove of DNA.

There is a controversial question about the hit necessary to initiate the aberrant methylation in cancer; the problem is related to its very early occurrence, before transformation of a healthy cell: thus, we "see" only the final outcome of this process.

The list of genes involved in tumourigenesis and silenced by methylation is becoming more and more extended. The actual field of research is addressed on defining a specific pattern of methylated genes for specific kind of tumours, the mentioned CIMP; this fundamentally implies that methylation is a non-random process, but also that methylation has a cause that should be searched for and identified. Actually our knowledge is still focused on the analysis of the final process that is the methylation status of genes; this kind

of approach has been improved with more and more sophisticated techniques, which allow to assess patterns of epigenetic modification in a gene-specific or genome-wide manner.

In normal cells, the active transcription of genes represents a hard obstacle to methylation setting in promoters; however these regions contain the molecular apparatus of the open configuration of the chromatin that is acetylated histones, transcription factors and coactivators. It is important to note that the regions flanking a promoter could have a closed and methylated structure without interfering with gene transcription. It is thought that unmethylated CpG islands are protected from the spreading of the surrounding methylation by the presence of not well characterized barriers. In a cancer cell, there could be a breakdown of these barriers with methylation then spreading into the CpG islands of promoter regions, that set up the closed and transcriptionally inactive configuration (Jones and Baylin, 2002).

In sporadic forms of cancer, methylation could affect the promoters of both alleles of a gene, while in the familial forms methylation always represents the second, not inherited, hit that leads to the complete silencing of the affected gene.

The number of epigenetically silenced cancer-related genes equals or exceeds the number of those inactivated by mutations. Thus, promoter hypermethylation affects genes involved in cell cycle (p16, p15, Rb, p14), DNA repair (BRCA1, hMLH1, MGMT) (Teodoridis et al., 2005), carcinogen-metabolism (GSTpi), cell-adherence (CDH1, CDH13), apoptosis (DAPK, TMS1) and many others (Esteller, 2002; Esteller, 2007).

Results obtained using different approaches indicate that there is a range of 100-400 promoter hypermethylated CpG islands in a given tumour and that this methylation profile differs among different tumour types. In particular, methylation of p16, p14 and O-6-methylguanine DNA methyltransferase (MGMT) was found in colorectal adenomas, while aberrant methylation of MLH1 is typical of endometrial hyperplasia. In contrast, the MAGE gene family has an opposite pattern of expression, because these genes are normally silenced through methylation in the healthy adult tissues and expressed only in

the testis. In many tumours, including lung, sarcomas, mammary and colon carcinomas, MAGEs are aberrantly demethylated and transcriptionally active, thus suggesting the potential use of this antigen as a target for cancer immunotherapy (Karpf and Jones, 2002; Ohman and Nordqvist, 2001).

Besides DNA methylation, epigenetic silencing of chromatin is related to the proteins involved in histone modifications and in chromatin structure, so we could speculate a deregulation also in these systems (Lund and van Lohuizen, 2004; Roberts and Orkin, 2004). There is a tight interdependence between DNA methylation and chromatin modification for DNA packaging: HDACs form a complex with both DNMTs and MBPs and deacetylated histones determine a more closed chromatin configuration. This implies a problem of hierarchy between DNA methylation and histone deacetylation, even though data obtained by treating cancer cells with 5-aza-dC seem to support the dominant role of methylation with respect to deacetylation induced by HDAC I and II (Jones and Baylin, 2002). Histone methylation makes the epigenetic regulation more complicated, because its impact depends on the specific Lys modified and on the number of residues bound. The hypermethylated promoters of cancer genes are enriched for enzymes known to catalyze Lys methylations. Subsequently di- and trimethylated H3K9 are recognized by chromodomain proteins, like HP1, which set the chromatin in a closed configuration. In human cells loss of DNMT1 results in a decreased level of H3K9me2 and H3K9me3, typical marks of silencing in heterochromatin and in euchromatin respectively (Fahrner et al., 2002).

Genes specifically silenced in a specific tumour could represent a biomarker: later I will discuss the first approaches to use GSTpi methylation detection in biological fluids and biopsies as a marker for detecting prostate cancer (Laird, 2003).

1.3 PROSTATE CANCER

The main function of the prostate is to produce seminal fluid. The prostate is made up of a fibromuscular stroma and epithelial glands, in which the adenocarcinoma occurs and which is composed of three kinds of cells: basal, luminal secretory and neuroendocrine. There are a few basal cells that secrete components of the basement membrane and a subset of these might also be the stem cells for the luminal epithelial cells.

The luminal cells express the androgen receptor and secrete in an androgen-dependent manner components of the prostatic fluid, including the prostate specific antigen (PSA). PSA is a serine protease of the kallikrein gene family, which is almost exclusively a product of the prostate cells and thus an exceptionally useful tumour marker for diagnosis of prostate cancer and for monitoring the effectiveness of a treatment. The stroma is composed of fibroblasts, smooth muscle cells, dendritic cells, nerves and some infiltrating cells, such as mast cells and lymphocytes. Some stroma cells are androgen-responsive and produce growth factors that act in a paracrine fashion on the epithelial cells. This stromal-epithelial crosstalk is an important regulator of the growth, development and hormonal responses of the prostate. The well-organized secretory glandular structure in the normal prostate becomes disrupted in prostate cancer.

Prostate cancer is one of the most frequent kinds of tumour among male population of the western world and accounts for 29% of all male deaths. Its development depends on numerous factors such as age, race, dietary and environmental factors, steroid hormones, family history etc., but undoubtedly, the most prominent risk factor is aging. In fact, approximately 75% of the clinically detectable prostate cancers are diagnosed in men between 50-70 years of age (Karan et al., 2003).

Another form of prostate disease is benign prostatic hyperplasia (BPH). This is due to the synthesis of a more potent form of testosterone, which stimulates the proliferation of

stromal and glandular elements. BPH is not a precursor or a premalignant lesion of prostate cancer (PCa), even though both require androgens for growth.

Prostate cancer develops in a stepwise manner going from normal prostate epithelium to androgen-independent cancer, across proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN), localized cancer and finally metastatic cancer (Feldman and Feldman, 2001).

The occurrence of an inflammatory process is an initial and common event in the disease's evolution and the factors typically associated with inflammation have been found to be involved in the development of prostate cancer. These include cell and genome damage, increased cellular proliferation a a tissue microenvironment rich in cytokines and growth factors (Palapattu et al., 2005).

The region characterized by inflammation is the peripheral zone of the prostate, containing proliferative epithelial cells (De Marzo et al., 2004). The inflammation could be considered the normal response of epithelial cells when infections, ischemia, or endogenous/exogenous toxins affect prostate tissue. The first reaction is the creation of a tissue microenvironment that promotes the recognition and repair of the cellular damage as well as the eradication of foreign particles, infected cells and irreparably damaged cells. Generally, two molecular events mark this phase: the synthesis of reactive oxygen and nitrogen oxide species by inflammatory cells (typically lymphocytes and macrophages), which can alter protein structure and function and cause lipid peroxidation, and the increased expression of carcinogen-detoxification enzymes, such as GST α , Cox-2 and GSTpi. GSTpi is normally expressed in basal cells, but not in luminal cells. When damage occurs, these cells produce high levels of GSTpi presumably in order to sustain the elimination of the dangerous species. GSTpi silencing exposes cells to genomic damage mediated by carcinogens and marks the transition between PIA and PIN or PCa (Figure 1.3.1).

1.3.1 Prostate Cancer: general characteristics

Although prostate cancer usually arises in the peripheral zone of the gland, it is generally multifocal and characterized by a marked molecular heterogeneity. A well recognized precursor of prostate cancer is the high-grade prostatic intraepithelial neoplasia (HGPIN), which consists of architecturally benign prostatic acini lined by cells that seem to be malignant.

PIN and PCa share some of the molecular changes conferring a growth or survival advantage. Similar to other types of epithelial cancer, PCa contains many somatic genomic alterations, including point mutations, deletions, amplifications, chromosomal rearrangements and changes in DNA methylation. But, unlike some carcinomas, for example colorectal and pancreatic, where specific oncogenes such as k-ras and p53 are usually mutated, PCa does show a high heterogeneity from case to case or even from lesion to lesion in a single case (DeMarzo et al., 2003).

The most famous tumour suppressor, p53, is rarely mutated in primary prostate tumours (10-20%) and this percentage increases in metastatic lesions. Similarly, inactivation of retinoblastoma (RB)1 and cyclin-dependent kinase inhibitor 2A (CDKN2A) genes is not so frequent in the early steps of tumourigenesis, but occurs at higher frequencies in metastatic and hormone refractory lesions. In contrast, alterations in NKX3.1 gene and Phosphatase and Tensin (PTEN) homologue gene are more widespread in prostatic tumours (Karan et al., 2003).

NKX3.1 encodes a prostate-specific homeobox gene that is essential for normal prostate development. NKX3.1 protein binds PSA promoter and represses the expression of the PSA gene and its expression decreases as cancer develops and evolves from a localized to an advanced stage.

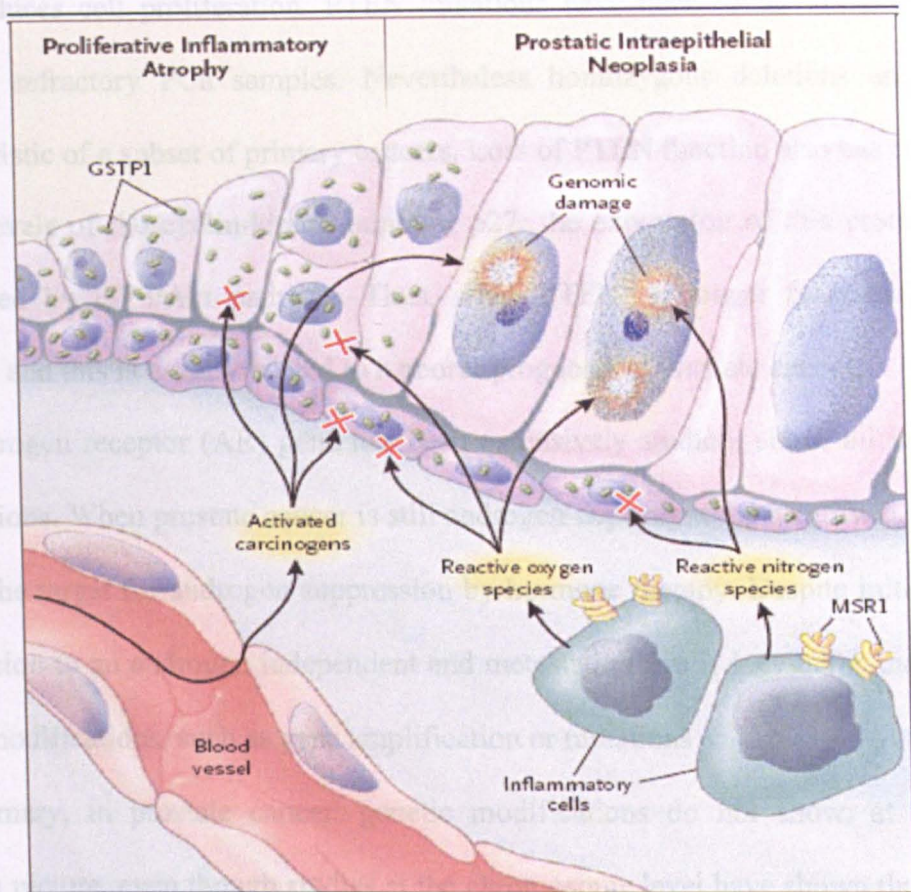


Figure 1.3.1 Loss of GSTP1 caretaker activity in prostate cells

Dietary carcinogens and oxidant carcinogens, elaborated by inflammatory cells, can be detoxified in basal epithelial cells and in cells undergoing proliferative inflammatory atrophy by GSTP1 (shown in its dimeric catalytically active form). Cells in the PIN zone, which are devoid of GSTP1, undergo genomic damage mediated by such carcinogens (MSR1, Macrophage Scavenger Receptor). (From (Nelson et al., 2003), modified)

PTEN is a tumour suppressor, because it is responsible for dephosphorylation and inactivation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) protein, which, in its active form, induces cell proliferation. PTEN mutations have been found in up to a third of hormone refractory PCa samples. Nevertheless homozygous deletions are a frequent characteristic of a subset of primary cancers. Loss of PTEN function also has consequences on the levels of the cyclin-kinase inhibitor p27: the expression of this protein is in fact suppressed by PIP3/Akt pathway. Thus, when PTEN no longer functions, p27 levels decrease and this is usually related to a poorer prognosis in prostate cancer.

The androgen receptor (AR) gene has been extensively studied, above all its therapeutic implications. When prostate cancer is still androgen-dependent for its growth, this receptor can be the target for androgen suppression by hormone therapy. Despite initial responses, progression to an androgen independent and metastatic form is inevitable, mainly because of AR modifications, such as gene amplification or mutations.

In summary, in prostate cancer, genetic modifications do not show, at the moment, uniform picture, even though studies at the chromosome level have shown that gains at 7p, 7q, 8q and Xq, and losses at 8p, 10q, 3q and 16q are very common, together with telomere shortening, which could also be evident in PIN lesions (Nelson et al., 2003).

1.3.2 Prostate Cancer and Epigenetic Modification

Prostate cancer is one of the most investigated tumours for methylation modifications acquired during its evolution. An aberrant epigenetic pattern has been found both in the early and late stages of the disease, but also in a significant fraction of high-grade PIN (Yegnasubramanian et al., 2004).

The growing list of papers published in the last few years on the epigenetics of prostate cancer underlined that this kind of tumour might be classified by a panel of methylation markers, including not only the hypermethylation in the promoter regions of several genes belonging to different pathways, but also a global hypomethylation (Yamanaka et al.,

2003; Li et al., 2005; Perry et al., 2006). As in other cancers, the role of global hypomethylation is relevant and in particular it affects the abundant repetitive sequences (such as the Long Interspersed Nuclear Element-1, LINE-1, retrotransposons) that are densely methylated in normal cells. Demethylation increases chromosomal instability, which is a mark of the advanced stage of this (and other) tumour types and their metastases. In addition, selected single copy genes become hypomethylated. Amongst these, is the gene encoding an urokinase plasminogen activator (PLAU) involved in tumour invasion and metastases. This shows extensive hypomethylation in hormone-independent cancer cells, together with the Cancer Associate Gene (CAGE, a novel cancer/testis antigen gene) and heparanase, an end- β -D-glucuronidase, which becomes highly expressed in cancer tissues compared to normal tissues.

In addition to this, more than 30 genes undergo aberrant hypermethylation in prostate cancer. These genes include classic and putative tumour-suppressors and genes involved in different cellular pathways, such as hormonal responses, tumour-cell invasion and/or tumour architecture, cell cycle control and DNA damage repair. For many of these genes, promoter hypermethylation is often the main mechanism determining their functional loss in prostate cancer. Inappropriate silencing of these genes can contribute to cancer initiation, progression, invasion and metastases (Figure 1.3.2 and Figure 1.3.3).

In general, it has been found, through clinical studies, that this “epigenetic catastrophe” usually involves the early stages of prostate cancer progression and confers an advantage to the clonal growth of the transformed cells. Genes usually methylated in the early stages of tumour development/aetiology belong to DNA damage repair and to transcriptional regulation pathways. Undoubtedly the most extensively studied gene in these classes is GSTP1 (Lin et al., 2001b), which is silenced through methylation in 90% of primary prostate tumour samples and in about 70% of PIN samples examined.

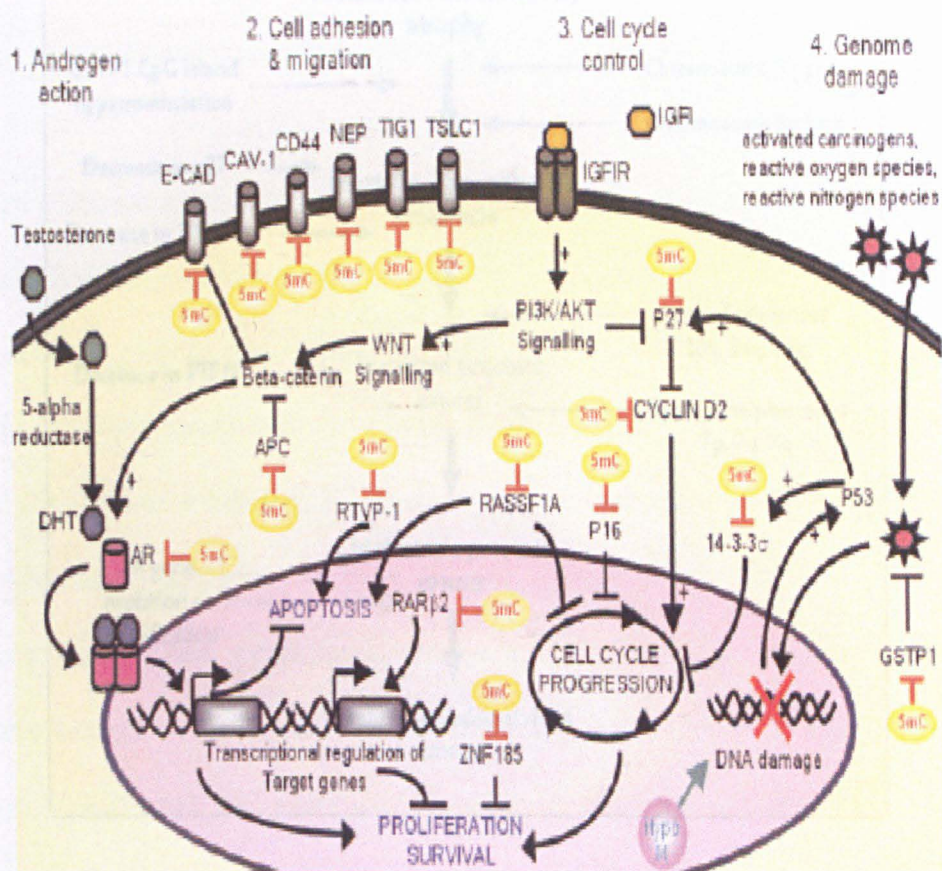


Figure 1.3.2 Cellular pathways affected by methylation and involved in prostate cancer development.

From (Perry et al., 2006), modified.

5mC indicates silencing mediated by methylated cytosine in the promoter of the corresponding gene.

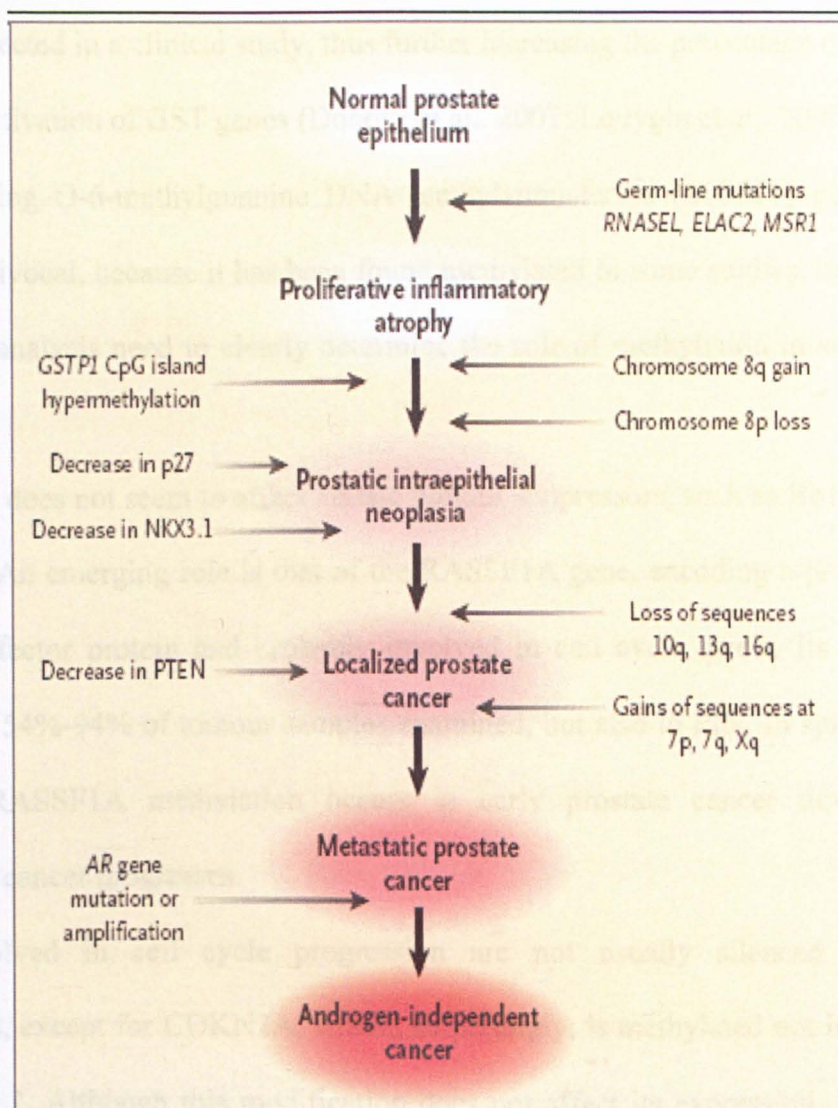


Figure 1.3.3 Molecular pathogenesis of prostate cancer

From (Nelson et al., 2003), modified

Very recently, methylation of the GST μ isoform has been found in about 54% of tissue samples collected in a clinical study, thus further increasing the percentage of tumours with a global inactivation of GST genes (Dobosy et al., 2007; Lodygin et al., 2005).

Data regarding O-6-methylguanine DNA methyltransferase (MGMT) gene are not so clear and univocal, because it has been found methylated in some studies, but not in others and further analysis need to clearly determine the role of methylation in silencing of this gene.

Methylation does not seem to affect classic tumour suppressors, such as Rb1, TP53 and the VHL gene. An emerging role is that of the RASSF1A gene, encoding a protein similar to the RAS effector protein and probably involved in cell cycle arrest. Its methylation is observed in 54%-94% of tumour samples examined, but also in PIN. In spite the few data available, RASSF1A methylation occurs in early prostate cancer development and increases as cancer progresses.

Genes involved in cell cycle progression are not usually silenced by epigenetic mechanisms, except for CDKN2A, which, surprisingly, is methylated not in the promoter, but in exon 2. Although this modification does not affect its expression, it is unique for prostate cancer tissue and could be useful as a marker (Li et al., 2004).

Late stages of prostate tumours, characterized by androgen-independent growth and by the metastatic process, depend on the inactivation of genes belonging to the adhesion system. Thus E-cadherin is usually methylated in cell lines and in high-grade prostate cancer, together with CD44, whose function is related to matrix adhesion and signal transduction.

In the androgen-independent stage of prostate cancer, the androgen-receptor (AR) gene becomes inactivated or deregulated in its function and it could be not expressed because of methylation of its promoter. The incidence of methylation-mediated AR silencing ranged from 0% to 20% in primary prostate cancers and from 13% to 28% in androgen-independent cancers. In contrast, methylation is the leading cause of the absence of the

estrogen receptors (ER α and ER β) and retinoic acid receptor β in the advanced stages of prostate cancer.

Although DNA methylation has been the most extensively studied epigenetic modification in prostate cancer, other chromatin changes can contribute to tumour transformation. Thus, histone modifications have a relevant role in allowing, or not, gene transcription, not only via their direct action on chromatin conformation, but also via the interaction with other regulating proteins and with DNMTs.

Histone acetyltransferases (HATs) affect a wide range of cellular processes by increasing transcriptional activity, for example that of the androgen receptor leading to an increased activity of this receptor, without the androgen stimuli. Conversely, HDACs, which are up-regulated 2-3 fold in PCa, especially androgen-independent PCa, are responsible for a closer structure of chromatin and seem to be involved in the decreased transcriptional activity of the vitamin D receptor. This prevents 1,25-(OH) $_2$ -vitamin D $_3$ exerting its cell cycle regulatory antiproliferative effects.

Another significant histone modification in prostate cancer is methylation: the EZH2 protein is responsible for Lys27 methylation on histone H3, which is a mark of transcriptional activation. EZH2 upregulated, especially in metastatic PCa (Schulz and Hatina, 2006).

The fundamental role of the epigenetic modifications, in particular DNA methylation, in prostate cancer initiation and progression has prompted investigators to use the most frequently methylated genes as markers for tumour detection. To be fit for this purpose, gene methylation detection methods need to be specific and sensitive and, at the same time, readily applicable to clinical specimens obtained through minimally invasive procedures.

Among all genes methylated in prostate cancer, GSTP1 shows the highest frequency and this occurs very early in tumour induction (Harden et al., 2003; Song et al., 2002).

Recently, analyses on different kinds of male bodily fluids have highlighted that GSTP1 hypermethylation may be useful for the early detection and diagnosis of PCa (Henrique

and Jeronimo, 2004; Hopkins et al., 2007). Sensitive and quantitative methods of detection help to establish this gene as tumour marker, while using very little tumour material for diagnosis. They can also detect tumour foci in apparently benign areas, especially because GSTP1 methylation is a positive signal surrounded by widely negative signals in each contaminant of the sample used.

Clinical studies have involved detections of GSTpi methylation in PIN, PCa tissues and metastases and, very rarely, in BPH (Nakayama et al., 2003), but also in blood, ejaculates and urine of the PCa patients (Gonzalzo et al., 2003). The possibility of finding GSTP1 methylation in these latter fluids could make it an instrument of diagnosis for PCa and for monitoring residual disease after curative surgery and therapy, instead of serum PSA, which lacks specificity because it does not distinguish BPH from PCa.

When we consider promoter methylation in body fluids as a cancer biomarker, the detection of a specific kind of tumour is extremely important. Specific prostate cancer detection could be compromised, because in males both liver and kidney tumours could show GSTP1 methylation, even if at much lesser extent than prostate cancer. As a consequence, the combination of the most frequently methylated genes in PCa should be considered as a tumour “fingerprint”, in order to improve PCa specificity and provide better diagnostic and prognostic information. Thus evaluation of various combinations of CpG island hypermethylation at GSTP1, APC, PTGS2, MDR1 (multidrug resistance 1) and RASSF1A promoters allows 100% sensitivity and >92% specificity (Tokumaru et al., 2004).

In summary, prostate cancer is undoubtedly characterized by widely distributed epigenetic modifications, which surely confer a growth advantage to the tumour.

The epigenetic causes of cancer in contrast to genetic causes allow the specific adoption of epigenetic therapies, in order to revert the aberrant modifications on DNA. For prostate cancer, this could be particularly suitable, because this kind of therapy could act simultaneously on androgen receptor function, cell adhesion and migration, cell cycle

control and genome damage and repair pathways, blocking tumour development at different steps and hence increasing the chances of success.

1.4 GLUTATHIONE-S-TRANSFERASE

Glutathione-S-Transferases (GST) represent a class of abundant detoxifying enzymes in both prokaryotic and eukaryotic cells. These proteins catalyze the attachment of the nucleophilic reduced peptide GSH to non-polar compounds containing an electrophilic carbon, nitrogen or sulphur atom. In general, we can summarize the reaction catalyzed as follows:



where R-X is the electrophilic substrate (Armstrong, 1997).

We can broadly distinguish soluble and membrane-associated enzymes. Among the former are the cytosolic and mitochondrial GSTs, sharing some similarities in three-dimensional folding. Microsomal GSTs, also referred membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) do not show any structural similarities to the other classes.

From a physiological point of view, GST enzymes confer protection to the cells against oxidative stresses, especially those resulting from oxygen radical attacks on membranes, nucleotides and fatty acid chains (in general, to biological macromolecules). However, GSTs exert their function also to protect against damage by exogenous electrophiles, such as environmental pollutants, industrial intermediates, pesticides and herbicides.

Much interest has arisen from the capability of GST to detoxify cancer cells from cytotoxic drugs and the link between GST activity/expression and anticancer drug resistance has been extensively investigated.

1.4.1 GST enzymes

Cytosolic GST enzymes fall into different classes, named alpha, mu, pi, theta, omega and zeta. They are 199-244aa in length. These different classes have a common evolutionary precursor gene, identified as the theta gene. During evolution, the alpha, mu, pi genes arose

by duplication of this gene and the most pronounced structural differences occurred in the catalytic region.

The enzymes form dimers of subunits belonging to the same class. This characteristic allows different homo- or heterodimeric isoenzymes and this widens the substrate spectrum of the catalyzed reactions. Allelic variants of each isoenzyme have also been described, and these can explain differences in the efficiency of the reactions.

In each class, mouse and human protein primary structures share about 40% identity, while no more of 25% identity has been found among different classes within a species.

GSTs have strong binding properties. In fact, besides catalyzing conjugation, reduction and isomerization reactions, cytosolic GSTs also bind, covalently and not covalently, hydrophobic non-substrate ligands. This contributes to intracellular transport, sequestration and disposition of xenobiotics and hormones.

1.4.1.1 Enzyme structures

GST structure consists of N-terminal and C-terminal domains, which totally differ for the secondary/tertiary structure. The N-terminal domain usually folds in four strands β -sheets and specifically binds GSH through electrostatic and hydrogen bonding interactions, while the C-terminal domain is constituted by α -helices and is particularly involved in the xenobiotic species recognition. To allow reaction catalysis, the xenobiotics lie in the crevice between the N-terminal and the C-terminal domains. This is the general structure of the soluble form of GSTs, and, although there are similar topologies, the structures differ in many details accounting for the specificity of each class. For example, mu and alpha class enzymes have a more constricted active site than pi, sigma and theta classes (Hayes et al., 2005).

I have previously mentioned that the functional form of GST enzymes is a dimer. This structure results from the interaction of two distinct subunits through a ball-and-socket model in alpha, pi and mu classes; in this model the side chain of a phenylalanine residue

(the ball) of the first subunit protrudes in the socket between the $\alpha 4$ and $\alpha 5$ helices of the second subunit. Up to now, catalytically active monomers have not been observed in mammals, but they have been described in non-mammalian species.

In order to activate the sulphur for nucleophilic attack, GST-GSH binding, occurring through hydrogen-bonding interaction on the dimer surface of the enzyme and the core $\beta\beta\alpha$ motif recognizing the γ -glutamyl portion of GSH, is the most conserved in all cytosolic enzymes. In contrast, the differences among the GSH binding sites of the various enzyme classes involve the interaction of the GST protein with the sulphur of the GSH peptide.

This common tridimensional structure does ensure catalysis of many different reactions, including, for example, nucleophilic aromatic substitutions, epoxide ring opening, reversible Michael additions, isomerizations, and peroxidase reactions. The substrate bonding occurs on the C-terminal domain II of the GST enzyme. This domain is typically characterized by differences in its architecture among the different GST classes. Nevertheless all GST enzymes share the capability of catalyzing the addition of GSH to 1-chloro-2,4-dinitrobenzene (CDNB), and this reaction is widely used as kinetic enzymatic assay to test the GST functionality. It is important to note that it does not discriminate the different GST classes (Habig and Jakoby, 1981).

GST alpha class is mainly expressed in the liver and is encoded by a gene cluster localized on chromosome 6p12. This cluster contains 5 genes, encoding for the 5 isoenzymes of the class (GSTA1-GSTA5). In turn, each isoenzyme is generally characterized by genetic polymorphisms, involved in transcriptional modulation of the genes. Among the different isoenzymes, A1, A2 and A4 are widely expressed in human tissues. GSTA1 is particularly active towards several carcinogens, including polycyclic aromatic hydrocarbons and epoxides, while the A3 isoenzyme is selectively expressed in steroidogenic tissues and plays a role in steroid hormone biosynthesis. In general, members of the alpha class possess high glutathione peroxidase activity and play an important role in protecting cells against the genotoxic effects of reactive oxygen species and the products of peroxidation.

Homozygous null GSTA4 mice have been characterized: they show an apparent normal phenotype, but are more susceptible to bacterial infections. Liver and brain contain increased mRNA levels for other GST classes (in particular, GSTA1/2, A3 and GSTM1) and for other enzymes involved in ROS detoxification.

Omega class. Two different isoenzymes characterize this not well-characterized GST, which do not catalyze the reactions typical of the other GSTs, except that for CDNB. They are abundantly distributed in the liver, macrophages, glial and endocrine cells.

Zeta class is particularly expressed in hepatocytes and renal proximal tubule cells and is mainly involved in the GSH-dependent transformation of α -halogenated acids. At the moment, the unique isoform of this class, GSTZ1, has been characterized as maleylacetoacetate isomerase (MAAI), which is in the catabolic pathway of phenylalanine and tyrosine. GSTZ1 null mice are prone to renal injury following phenylalanine and tyrosine overload and die if provided with drinking water containing phenylalanine.

Theta and sigma classes. GSTT1 gene is located on chromosome 22q11.2. One of the reactions catalyzed by this class is the conjugation of halomethanes in human erythrocytes, but GSTT1 substrates also include industrial and environmental chemicals. Recent studies have found GSTT1 expressed particularly in prostatic tissue, but there is little insight on the consequences of this overexpression.

GST sigma encodes the hematopoietic, GSH-dependent, prostaglandin D₂ synthase. The phenotype of the KO mice is related to allergic reactions, which are weaker than in WT mice.

GST mu class is characterized by 5 different isoforms (GSTM1 → GSTM5), which, in turn, contain different polymorphisms. GSTM genes are located in tandem on chromosome 1, but they can be differentially expressed in human tissues. In particular, GSTM1 has been found in liver, stomach and brain, while isoforms GSTM2-GSTM5 have uniquely an extrahepatic expression. GSTM1 is involved in detoxification of numerous electrophilic substances including carcinogens, and, probably, of lipid peroxidation products. GSTM2

and M3 have brain localization. GSTM2 has been identified as prostaglandin E: in the brain it binds GSH to redox cycling products of dopamine, thus allowing their detoxification: in an unbound form, these products can contribute to the processes involved in neurodegenerative diseases, such as Parkinson's disease and schizophrenia. Therefore the main role of GSTM2, especially of its B allelic form, could be protection against neurodegeneration. KO mice for GSTM5 have been generated, but, at the moment, no clear phenotype has been described.

GSTpi class has been the most extensively studied, in particular for its well-recognized role in carcinogenesis. From a physiological point of view, GSTpi was first identified as placental isoform but was subsequently found in many tissues at variable levels. It is the most ubiquitous and highly expressed GST class in mammals. Its expression is inducible by exogenous agents including chemotherapeutic agents and the overexpressed protein is a mechanism of resistance to anticancer therapy of a wide range of human tumours.

GSTP gene is located on chromosome 11 as single unit, but it is highly polymorphic and the different alleles have different efficiencies in catalytic activity.

KO mice for GSTP isoforms 1 and 2 do not have an aberrant phenotype or an increased incidence of any kind of tumours; but when treated with TPA or 7,12-dimethylbenzanthracene (DMBA), a significantly higher number of skin papillomas were induced than in wild type mice (Gate et al., 2005). Similarly, treatment with tobacco smoke components increased the number of lung adenomas formed. This peculiar sensitivity of lung and skin to GSTpi absence could relate to the fact that GSTpi is the most abundant GST form in lung and skin of the humans (Henderson and Wolf, 2005; Hayes et al., 2005).

1.4.1.2 GSTpi hypermethylation

As I described before, an aberrant DNA methylation could be now considered a hallmark of cancer, together with those previously analyzed by Hanahan D, and Weinberg R.A. in Cell (Hanahan and Weinberg, 2000).

There have been numerous attempts to try to associate a specific marker of methylation to a specific kind of tumour. In this regard, hypermethylation of the human GSTP1 gene represents a useful and specific tool to particularly identify prostate cancer cells, because the 5'-region of GSTP1 gene contains a CpG island that is the target of an aberrant hypermethylation during tumourigenesis. This event occurs very early in tumour development and this made it difficult to evaluate the kinetics of the silencing process, particularly when a cell line is used for such analyses.

Nevertheless, studies on prostate tissues highlight that there is a clear difference in methylation status between benign tissues and tumours, and that GSTP1 methylation is involved in a more general "epigenetic catastrophe" that targets many kind of genes. Both alleles of the gene are affected, resulting in the complete absence of the protein in the cells. We can consider GSTP1 a "caretaker" gene, because loss of the protein renders prostate cells vulnerable to genome damage (generally associated with chronic prostate inflammation) via repeated exposure to carcinogens, which, in turn, promotes transformation to high-grade PIN and prostate cancer.

GSTP1 is an autosomal gene located on chromosome 11q13; it spans about 4kb and comprises 7 exons and 6 introns, encoding for a 715b mRNA transcript. The GSTP1 promoter is located in a 1.5kb CpG island (from ~400bp upstream to 800bp downstream of the transcription start site), that also spans exons 1-3: its core region contains two Sp1 sites, a consensus AP-1 site and a TATAA box (Rushmore and Pickett, 1993). Detailed methylation analysis of the entire gene showed that, in normal prostate tissues, the CpG island is completely unmethylated; however, beyond the CpG island region, from intron 4 to the 3'end of the gene, there is extensive methylation in both normal and prostate cancer tissue, even though this does not affect gene expression (Lee et al., 1994). In contrast, in prostate cancer tissues, adjacent promoter CpG dinucleotides are fully methylated and this correlates with the loss of GSTP1 enzyme expression. Hypermethylation was found in both

the top and the bottom strands of DNA, while no mutations affecting GSTpi expression have so far been reported (Millar et al., 1999; Esteller et al., 1998).

The GSTP1 gene modification by methylation is a step by step process, which starts with the transcriptional silencing of the gene. Genes encoding GSTs are characteristically expressed at very low levels in many tissues, until induced via increased transcriptional activity, by exposure to oxidants and electrophiles. In the absence of an inducing exposure, low levels of GSTP1 transcription might render its CpG island vulnerable to de novo DNA hypermethylation. In fact we actually have to think of methylation setting as a consequence of a broken balance between methylated and unmethylated status, regulated by the transcriptional level of the gene.

Probably when transcriptional activity becomes lower, de novo methylation is favoured and begins to spread across the promoter. The transcriptional silenced gene is targeted by the methyl binding protein MBD2 at methylated cytosines, which contributes to the assembly of a protein complex involving histone deacetylases and DNA methyltransferases. This establishes a closed chromatin conformation, that may be responsible, together with methylation of H3K9, for the maintenance and persistence of the silenced state in the subsequent tumour cell generations, (Stirzaker et al., 2004).

The methylation spreads across a boundary element at the 5'end of the CpG island. This region is marked by a repeated ATAAA sequence, which in turn is adjacent to an Alu repeat in 3'; such sequence boundary has not been found at the 3'end of the GSTP1 island. In normal prostate tissue, methylation is confined to the Alu repeats and no modification occurs both in the ATAAA repeat and in the CpG island. It is not clear whether the ATAAA repeat is itself the boundary element or whether it is fortuitously located 5' or 3' to the actual barrier sequence, which protects the CpG island from methylation. However it does not appear to contain binding sites for proteins, which could represent a physical obstacle to the moving of methylation and also the Sp1 binding site does not seem to be involved in protection against de novo methylation.

In prostate cancer cells, this marked methylation boundary is disrupted or bypassed as the entire CpG island, on both alleles, is methylated, following the transcriptional silencing of the gene (Millar et al., 2000).

Undoubtedly, reduced expression of the GSTP1 gene likely plays the main role in its silencing and could represent the starting point for the chemical modifications of DNA and histones in its promoter which “lock” the chromatin in this region.

1.4.1.3 Besides the GSH transfer catalysis: regulatory functions of GSTmu and pi

Earlier I described the GST catalysis of GSH ligation to endogenous and exogenous electrophiles, presumably in order to allow their detoxification. Although the catalytic efficiencies for many of the substrates are low, GSTs have, as we have seen before, pleiotropic substrate specificity. The fundamental role of the GST family in cell defences is further widened by data showing its involvement in a regulatory (and non catalytic) binding activity to the stress-activated kinases, such as the c-Jun-N-terminal kinase (JNK), which links stress stimuli to apoptotic response. At the moment this function has been demonstrated for GSTmu and GSTpi, the latter being typically overexpressed in many kinds of tumours, but not in prostate cancer (McIlwain et al., 2006).

GSTpi and mu exert their regulatory function as monomers, in contrast to their dimeric structure, by which they catalyze the conjugation of GSH to electrophiles.

Based on this regulatory activity, GSTpi and mu have been often referred as “ligandins”. JNK is a member of the mitogen activated stress kinase family (MAPK), which also includes the extracellular signal regulated kinase (ERK) and p38-MAPK. JNK was initially identified as a protein involved in the cellular response to environmental stresses (UV light, H₂O₂, osmotic shock and withdrawal of growth factors), pro-inflammatory cytokines and interleukins. JNK regulates c-jun (which belongs to the transcription factor complex AP-1), by binding to c-jun N-terminus and phosphorylating it at Ser-63 or Ser-73.

In non-stressed cells, GSTpi binds the NH₂-terminal region of JNK and so it prevents JNK being phosphorylated and hence catalytically active. Under conditions of oxidative or chemical stress, GSTpi releases JNK and the two unbound proteins exert their specific and catalytic functions. GSTpi will form a dimer to detoxify the dangerous molecules, while JNK, in its phosphorylated form, will, in turn, phosphorylate c-jun, which acts as transcription factor in the apoptotic pathways, if a prolonged or high level exposure to stress signals occurs (Wang et al., 2001).

A similar pattern applies to GSTmu, which exerts its regulatory function by binding the apoptosis signal-regulating kinase1 (ASK1) to form a GSTmu:ASK1 complex. In conditions of oxidative stress, or, more specifically, of heat shock, GSTmu oligomerizes, allowing the release of ASK1 and the subsequent induction of apoptosis, generally through the activation of downstream kinases (Cho et al., 2001). It is important to underline that the fate of the cell (i.e. either proliferation or apoptosis) after the activation of these stress-induced pathways, will be dependent upon the concentration and exposure time of the stress. In some cases, transient or low level exposure can induce cell proliferation, while prolonged or high level exposure can induce apoptosis (Gate et al., 2004).

To complete the description of GSTpi activity, it should also be considered that the GSTpi catalytic property can be associated with its regulatory function. In fact GSTpi plays an important role in the glutathionylation of the oxidized and inactive form of 1-cys-peroxiredoxin (1-cys Prx). The GSH binding to the catalytic cysteine of 1-cys-Prx leads to restoration of its peroxidase activity, thus enabling it again to respond to oxidative or nitrosative stresses.

1.4.1.4 Mitochondrial and MAPEG enzymes

Glutathione-S-Transferases K. is widely and uniformly expressed in human tissues and is the only GST enzyme found in mitochondria. New data show that it is also located in peroxisomes, suggesting that it might specifically be involved in β -oxidation of fatty acids,

either through its catalytic activity or via interaction with a membrane pore. As with the other soluble members of the GST family, GSTK has a high activity for CDNB.

Considerably less is known about the structure and the mechanism of the microsomal GSH transferases that are involved in xenobiotic metabolism. In contrast, there is more insight into their role in the production of eicosanoids. Six human MAPEGs have been identified and, based on their primary structure, they fall in four subgroups. These enzymes are characterized by a trimeric structure, each subunit consists of five membrane-spanning regions, at least two of which are likely to be α -helices perpendicular to the membrane surface. The N-terminus of the enzyme appears to be located on the luminal side of the endoplasmatic reticulum, while the C-terminus and the active site face the cytosol.

Three MAPEG enzymes are involved in eicosanoid synthetic pathway, and are identified as leukotriene C4 synthase (LTC4S), 5-lipoxygenase activating protein (FLAP) and as prostaglandin E2 synthase 1 (PGES1). There is no evidence of their involvement in oxidative stress responses (Hayes et al., 2005).

1.4.2 GSH

Glutathione (GSH) is a water-soluble tripeptide composed of the aminoacids glutamine, cysteine and glycine. It serves to maintain a reducing cellular environment and this function is so important that GSH is widespread in plants, mammals, fungi and some prokaryotic organisms. In some human tissues, it attains millimolar concentrations, especially where it exerts a detoxifying role, such as in the liver and brain.

This tripeptide can exist intracellularly in either an oxidized (GSSG) or reduced (GSH) state. Maintaining an optimal GSH:GSSG ratio in the cell is critical to survival, hence there is tight regulation of the system. When alterations in the redox balance occur by exposure to ROS, dose-dependent changes in GSH:GSSG ratios can potentially influence a number

of target proteins, by causing oxidation and disulfide exchange reactions at specific cysteine residues.

GSH deficiency puts the cell at risk for oxidative damage; furthermore, many different pathologies show an imbalance of the GSH levels.

GSH synthesis is inhibited by the phosphorylation of γ -glutamylcysteine synthase (γ -GCS). However GSH itself suppresses its own synthesis when high levels are reached in the cell (Townsend et al., 2003).

1.4.3 GST and CANCER

The role of GST polymorphisms in cancer susceptibility and the increased GST levels in determining anticancer drug resistance have been recently described (Townsend and Tew, 2003; Waxman, 1990).

In general, absent GST enzymes may result in a poorer elimination of the electrophilic carcinogens, which increase the risk of somatic tumourigenic mutations on DNA.

A null genotype for GSTM1 or GSTT1 classes can be found in humans and, in particular, GSTT1null is related to a higher risk of acute or chronic myelogenous leukaemia, while the GSTM1 null genotype was associated with an increased risk of prostate cancer (Katoh et al., 2008; Rebbeck et al., 1999).

The GSTpi picture is undoubtedly more diverse: as with other GST enzymes, GSTpi is overexpressed in a wide variety of tumours, including ovarian, non-small cell lung cancer (NSCLC), breast, colon, pancreas and lymphoma, usually as a response to chemotherapy. Among the different GSTP polymorphisms, GSTP1A has been reported to play a role in the acquisition of resistance to cisplatin via the formation of platinum-GSH conjugates. Patients homozygous for the GSTP1B allele have a reduced capacity to detoxify platinum - based anticancer agents, thus making this phenotype favourable in terms of response rate. Individuals positive for the GSTP1C allele seem to have a lower incidence of breast cancer. Except for prostate cancer, an elevated GSTP expression is generally associated

with a more aggressive tumour, a poorer prognosis and resistance to chemotherapy (McIlwain et al., 2006; Dang et al., 2005).

When we consider these increased levels of GST activity in cancer, we think not only of an increased capacity to detoxify drugs, but also of the regulatory roles of GSTs as monomers, especially GSTpi and mu. This non-enzymatic role of GSTpi also has a direct relevance in drug-resistant tumours. In fact, as an endogenous switch for the control of the signalling cascade pathway, elevated expression of GSTpi can alter the balance of the regulation of kinase pathways during treatment, thereby conferring a potential selective growth advantage.

There is much data on the effects of anticancer drugs on GST levels in cancer cells. Some drugs (chlorambucil, melphalan, nitrogen mustard, acrolein, ethacrynic acid) are direct substrates of GSTs and resistance strictly depends on increased levels of GST, in particular GSTpi. In general, a common characteristic of these drugs is the electrophilic nature of their active cytotoxic moieties, so they can be directly inactivated through catalytic conjugation to GST through a thioether bond formation. Other common anticancer drugs have not been characterized as GST substrates, even though the corresponding resistant cell lines show increased levels of this detoxifying protein. Typically the drugs include antimetabolites, antimicrotubule drugs, topoisomerase I and II inhibitors, mitomycin C, adriamycin, cisplatin and carboplatin (Tew, 1994). Many of these drugs induce apoptosis via activation of the MAP kinase pathways, specifically JNK and p38. Due to the fact that GST also acts as an inhibitor of the MAP kinase pathway, elevated GST levels could in turn determine an increased resistance to apoptosis. Cisplatin is an example of a drug whereby JNK activity is required for maximal cytotoxicity: inhibition of the JNK signalling pathway leads to a decrease in cisplatin-induced apoptosis, while c-jun overexpression increases cell sensitivity to this drug (Townsend and Tew, 2003). Thus, increasing GST levels is a generalized means of cell defence against anticancer drugs and, as a consequence, GST could represent a target to design new molecules that could

specifically kill tumour cells, instead of exerting a non-specific cytotoxic effect on all tissues, including the normal tissues, which usually determines the toxicity of the classic therapeutic regimens.

1.5 ANTICANCER THERAPY

The history of anticancer drug therapy covers the past century and offers many insights to develop new and more specific drugs to eliminate cancer cells. The first molecules were discovered quite serendipitously from their cytotoxic effects hematopoietic cells. Only later were the efforts applied to solid tumours. In general, the mechanism of action of these drugs was unknown and usually only identified and more extensively studied after testing their efficacy on patients. The first example was a mustard gas compound, which alkylates purine bases in DNA, leading to crosslinking of the strands and then apoptosis. This was followed by the discovery of the antifolates, such as methotrexate, purine analogs and other alkylating agents.

The first drug combination treatment took into account the use of the known cytotoxic drugs in order to circumvent cancer resistance and relapse after the treatment with a single agent. Another significant problem arisen during clinical trials was the acute and long-term toxicity of chemotherapy, which affected almost every organ of the body.

As molecular and genetic studies tried to understand the biology of tumour cells compared to normal cells, the possibility of a targeted therapy against deregulated markers arose. The targets included growth factors, signalling molecules, cell cycle proteins, modulators of apoptosis and molecules promoting angiogenesis. The first successful drug developed intentionally against a molecular marker was Imatinib, as inhibitor of the BCR-Abl kinase, which is relevant in chronic myeloid leukaemia.

Another target is the epidermal growth factor receptor (EGFR) both at its ATP-binding moiety level (using Gefitinib or Erlotinib) and at the extracellular domain level (using Cetuximab).

The central role of angiogenesis in allowing tumour proliferation and metastasis has made antiangiogenic therapy an attractive field for anticancer strategies.

Since then, new molecules have been synthesized against the vascular endothelial growth factor (VEGF) receptor, and, despite some clinical failures of this approach, new strategies are exploring the combination of antiangiogenic drugs and established cytotoxic molecules.

Thus, the perspectives of combination therapy have changed: from being an empirical set of cytotoxic drugs, to a more effective strategy based on the discovered molecular markers for particular kinds of tumours (Dancey and Chen, 2006). In addition, more clinical trials are now designed with the aim of identifying subsets of patients that are more likely to respond to certain drugs, thereby avoiding the needless cost and toxicity of an ineffective treatment.

1.5.1 Systemic Treatment for Prostate Cancer

As already outlined, prostate cancer (PCa) is the most common non-cutaneous cancer in the western world. Its incidence increases with age and it is also related to dietary and environmental factors. The early detection of transformed prostate cells benefitted from the advent of serum prostate specific antigen (PSA) testing, which has enabled a more extensive screening of the male population.

Many prostate cancers initially require the presence of circulating androgens that influence the proliferation and maintenance of the secretory prostate epithelium. Because cell turnover in the prostate is relatively slow, the multistep evolution from a restricted form to epithelial cells to invasive cancer is estimated to span decades. In the localized disease stage, patients are treated with radiation therapies and radical prostatectomy, which certainly allows a full pathological assessment of the tumour, but, at the same time, can have significant side-effects, such as impotence and urinary incontinence. Even now, the optimal therapy for early stage prostate cancer is not clear, especially because of the need for a long follow-up after randomized clinical trials.

In contrast, considerable efforts have addressed the advanced stages of prostate cancer. The most widespread therapeutic approach is androgen withdrawal, which usually results in tumour responses in 80-90% of patients. Androgen ablation therapy is very effective in androgen-dependent cancer and nowadays, instead of orchiectomy, this kind of therapy is performed through pharmacological methods, using a combination of androgen receptor antagonists with a gonadotropin-releasing hormone (GnRH) inhibitor. AR antagonists also prevent androgens produced by the adrenal glands from binding to AR in the prostate (Horwich, 2006).

Androgen ablation therapy fails because of the development of the androgen-independent stage of disease: intermittent administration was used in attempts to slow down this disease progression, but these trials did not produce clear results. Resistant tumours accumulate stepwise changes in DNA structure and gene expression, that provide a selective survival and growth advantage to the cells, which then no longer need hormone for growth (Feldman and Feldman, 2001).

In the last few years different therapeutic options for hormone refractory prostate cancer have been investigated.

Chemotherapy is being explored and the first survival benefit has come from treatment with docetaxel as first line therapy. More encouraging results have since been obtained by combination treatment, for example with prednisolone (causing adrenal suppression). Among other cytotoxic drugs, platinum derivatives, such as satraplatin, showed promising preclinical results against taxane-resistant cells, thus supporting subsequent clinical applications. Actually, the newest approach, as already mentioned, is to target those molecular markers which specifically distinguish cancer cells from normal cells and generally this is in association with cytotoxic chemotherapy.

Antiangiogenic therapy seems to have potential, because plasma levels of VEGF increase from healthy controls to patients with localized or metastatic prostate cancer. Bevacizumab, a VEGFR inhibitor, has been enrolled in combination with docetaxel against

metastatic hormone refractory prostate cancer (HRPC), leading to a substantial prolongation of life compared to administration as a single agent.

Small molecule inhibitors have been specifically designed to target those proteins which contribute to tumour growth and progression in prostate cancer. Thus atrasentan is an antagonist of the endothelin-A (ET-A) receptor, while imatinib is an agent that inhibits the tyrosine kinase activity of the platelet-derived growth factor receptor (PDGF), which is abundant in metastatic prostate cancer. Both atrasentan and imatinib have been used in clinical trials in combination with docetaxel.

A new approach comes from nucleotide-based targeted therapy, based on the use of antisense oligonucleotides (ASOs) that are complementary to a selected gene's mRNA. These targeted RNAs show an increasing level in transformed prostate cells and are usually related to cancer progression and aggressiveness, but also to the acquisition of drug resistance. At the moment, genes targeted for silencing are clusterin (which suppresses apoptotic cell death in response to androgen withdrawal and whose levels increase with the Gleason score), ribonucleotide reductase (RNR, an important enzyme for cell division and tumour growth, whose R2 subunit is often overexpressed in tumours, thus promoting drug resistance), but, above all, HSP27, a heat shock protein that is strongly induced under conditions of cell stress. The levels of HSP27 increase as prostate cancer becomes more and more aggressive and metastatic. HSP27 may serve as therapeutic "hyper-node", a target situated at the centre of many pathways involved in regulating cellular responses to treatment-induced stress, so its inhibition would silence multiple survival pathways at once. At the moment, ASOs against the proteins described above are in phase I/II clinical trials, usually in combination with docetaxel (Hadaschik and Gleave, 2007).

From a therapeutic point of view, of great potential, but still poorly investigated in prostate cancer, is aberrant DNA methylation which is characteristic of the disease initiation and progression and affects many different pathways (Li et al., 2005). To date, clinical trials of agents reverting genomic DNA methylation have obtained satisfying results only in

haematological malignancies. Actually, one such agent, decitabine, has undergone a phase II clinical trial for the treatment of hormone refractory metastatic prostate cancer, but serious side effects, including myelosuppression and the promotion of malignant transformation of genes, through induction of global DNA hypomethylation, have compromised its further development as a single agent. New approaches should consider the combination of methylation-modifying drugs with histone deacetylase (HDAC) inhibitors and conventional chemotherapeutic agents. To this purpose, GSTpi, whose epigenetic inactivation marks one of the earliest steps of carcinogenesis of the prostate, could represent a good target for reverting-therapies, in order to restore GSTpi-mediated detoxification processes and hence to attenuate genome-damaging stresses.

1.5.2 GST as a target for anticancer therapies

The first consideration when we think of GST as target for anticancer therapy is its overexpression in tumours, which supports the idea of a greater specificity of a possible treatment against tumour than normal cells. Considering the multiple roles of GST in cells, different approaches could target GST in its detoxifying activity, or in its modulatory role of the stress kinases or in its GSH binding activity. As I described earlier, increased GSTpi levels develop as a mechanism of resistance, especially after treatment with cytotoxic drugs that are GST substrates. Using a specific inhibitor of GST, the resistant phenotype should be reverted. For this purpose, the first inhibitor designed to lower GST levels in tumour cells was ethacrynic acid (EA), which acts as a non-competitive inhibitor of GSH for GST binding as well as depleting the GSH cofactor by forming an EA-GSH conjugate. Binding to both GSH and GST serves to inhibit enzyme activity. EA has been reported to potentiate the cytotoxic effects of chlorambucil and melphalan, but the lack of specificity against a particular class of GST (EA inhibits GST α , μ , π) and its side effects (EA has diuretic properties) have reduced its therapeutic value (Townsend and Tew, 2003).

Another GST inhibitor, specific for class pi, is TLK199. This drug has been demonstrated to improve the sensitivity of different tumour models to anticancer drugs (Townsend et al., 2005). However treatment with TLK199 causes a myeloproliferative effect in mice and this could be explained as a consequence of the disruption of the interactions between GST and JNK: the increased levels of free JNK could lead to an increased cellular proliferation in the bone marrow.

As regards the synthesis of prodrugs that can be activated, hopefully in a specific way, in the tumour cells, GST P1-1 represents a promising target, because its expression is enhanced in many tumours that have a poor prognosis or are resistant to classic cytotoxic drugs. The lead compound of this class has been TLK286, which is cleaved in the cell into a GSH analogue and nitrogen mustard, that then alkylates cellular nucleophiles. Clinical trials of TLK286 have shown limited adverse side-effects but significant antitumour activity and/or disease stabilization in patients with various advanced malignancies that were usually resistant to platinum and irinotecan. This supports its further development in the late stages of clinical experimentation (Townsend and Tew, 2003; Townsend et al., 2005).

New prodrugs have been designed in order to release NO after a GSTpi catalyzed reaction; they also induce apoptosis, after activation of p38, JNK and ERK proteins (McIlwain et al., 2006).

Many tumours overexpress GSTpi and prostate cancer is thus unique in that it lacks the GSTpi detoxifying activity, which could make this tumour particularly susceptible to drugs that are inactivated by GSH binding. However, as I hinted above, treatment with these drugs has no significant clinical effect on hormone refractory PCa and this could open the path to new therapeutic strategies.

The methylated GSTP1 gene represents an early and well recognized marker of malignant transformation of the prostate. Thus its re-expression could be an advantage both for cell

protection against stress and for the possibility of using prodrugs, that are activated intracellularly by GSTpi.

1.5.3 Molecules Reverting Epigenetic Modifications of DNA

Gene silencing through epigenetic mechanisms represents a reversible biological modification, which can be targeted for treatment of human cancer (Issa, 2007). This is undoubtedly an advantage compared to gene inactivation through mutations or deletions and, in the last years, there has been an increasing interest in developing therapeutic strategies capable to reactivate epigenetically silenced genes. At the moment, targets of epigenetic therapies are DNA methylation and histone modification, in particular histone deacetylation, that, as I have described previously, means transcriptional silencing.

The first specific inhibitors of DNA methylation were the nucleoside analogs 5-azacytidine (5-aza-CR) and its deoxy- derivative, 5-aza-2'-deoxycytidine (5-aza-dC); initially they were classified as differentiating agents rather than demethylating molecules. They are still extensively used and were recently approved by the Food and Drug Administration (FDA) for use in haematological malignancies. More recently, the use of antisense oligonucleotids, directed against the DNMT1 mRNA, was investigated, aimed to specifically inhibit DNMT and to avoid the toxic effects of nucleoside analogs.

Some demethylating properties were serendipitously discovered for drugs already used for different purposes in clinic: among them, procainamide and procaine, respectively an anti-arrhythmic drug and a local anaesthetic were shown to be able to revert promoter gene methylation.

Epigenetic silencing is almost universally associated with histone deacetylation, which is catalyzed by at least three classes of HDACs in human cells. The HDACs are partly redundant in function and a growing series of small molecules has been designed to inhibit their activities either globally or more specifically. Because it has been demonstrated that DNA methyltransferases and histone deacetylases can interact each other, a variety of trials

are now combining HDAC and DNMT inhibitors, in order to reduce some of the side-effects associated to DNA methylation inhibition, while still achieving an effective level of gene reactivation (Lyko and Brown, 2005).

Treatment with “epigenetic” drugs may have consequences that are not only uniquely related to the reactivation of genes associated with tumour transformation, such as tumour suppressors. In fact the first epigenetic strategies were based on the simplified concept that reactivation of these genes would surely lead to tumour growth arrest. In this respect, the regulation (i.e. demethylation) of the p16 promoter provides an example of this approach; but this causal relationship has not been always demonstrated (Karpf and Jones, 2002).

An important consideration for strategies that seek to reactivate gene expression is the phenomenon of re-silencing: both nucleosides analogs and non nucleoside inhibitors do not block DNMT1 synthesis, but trap it on DNA, thus implying that continuous and effective re-expression could be achieved only with prolonged treatment.

In addition to gene expression changes that occur as a direct consequence of reversing promoter methylation status in a specific gene, indirect gene expression changes in other genes may also result. This can depend on the relationship existing among different proteins, especially if belonging to the same signal transduction pathway. An example is given by the activation of the interferon signal transduction after treatment of colon cancer with 5-aza-dC: the data demonstrated that interferon pathway genes are not each individually regulated by methylation.

So therapeutic strategies should consider not only the re-activation of the target methylated genes, but also more general consequences on the pattern of gene expression, in order to understand the global effects obtained after treatment with these demethylating molecules. Examination of gene expression changes in a bladder cancer cell line compared to normal fibroblasts showed that approximately 60% of the genes induced by 5-aza-dC did not contain promoter CpG islands, thus supporting the notion that 5-aza-dC treatment regulates the expression of many genes indirectly. At the same time, not every promoter-methylated

gene is re-activated after treatment with demethylating agents. Approaches aimed at evaluating the global genomic changes in methylation demonstrated that about 600 CpG islands (out of 45000 in the human genome) may be aberrantly methylated in individual human tumours and that 5-aza-dC treatment led to gene reactivation of about 40% of them. This could depend on different factors: first of all, a gene becomes re-expressed following promoter hypomethylation, only if the treated cells have transcription factors competent for the binding and activation of the unmethylated gene promoter. However it is also possible that the majority of genes are not activated by 5-aza-dC treatment because chromatin structural alterations are dominant over methylation for silencing (Karpf and Jones, 2002). This supports the need to combine demethylating drugs with molecules reverting histone modifications. But the use of histone demethylase inhibitors in epigenetic therapy is not ideal, because the methyl groups on histones can result in either transcriptional activation or silencing, whereas histone deacetylation always means silencing. In the last few years, there has been much interest in the development of molecules targeting HDACs (Yoo and Jones, 2006).

The main problem of the demethylating therapy is its lack of specificity against a particular set of genes, which results in a global DNA hypomethylation, linked in turn to genomic instability, elevated rates of mutations and the activation of transposable elements. Moreover, the combination with HDAC inhibitors does not resolve this problem and the therapy still affects the global genome. Nevertheless, because epimutations rarely appear in healthy tissues and because epigenetic drugs act on actively dividing cells, epigenetic therapies could more efficiently affect tumour cells rather than normal cells. This makes feasible the use of these drugs for anticancer therapy and opens a window for their combination with new or classic cytotoxic molecules, which could take advantage from the altered expression state of the genome, to exert their killing effects (Plimack et al., 2007).

DNA methylation inhibitors fall into three classes:

- nucleodides inhibitors,

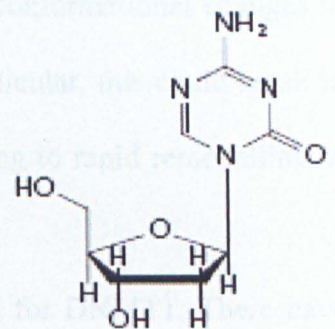
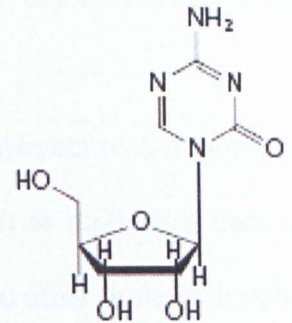
- non nucleoside inhibitors,
- rationally designed inhibitors.

1.5.3.1 Nucleoside inhibitors

The prototype molecule of this class is

5-azacytidine (5-aza-CR, higher panel):

it was discovered as cytodifferentiating agent by Jones et al. in the early 1980s (Jones and Taylor, 1980): in experimental conditions, 5-azacytidine allowed myogenic conversion of cultured mouse embryo cells, and this phenomenon was related to inhibition of methylation. This drug, together with its deoxy- analog, 5-aza-2'-deoxycytidine (5-aza-dC, lower panel), has a modified cytosine ring. They are incorporated into DNA by DNA polymerase, during the active replication.



Before this step, they are metabolized by kinases that convert the nucleosides into nucleotides for incorporation into DNA and/or RNA (Momparler, 1985). In fact ribonucleotides, such as 5-aza-CR and zebularine (which I will describe later) are phosphorylated by uridine/cytidine kinase and other kinases to generate the corresponding mono-, di-, and triphosphates, which ultimately end up in RNA. The effects of their incorporation into RNA have not yet been well studied, although some data suggest that this results in ribosomal disassembly, defective tRNA function and inhibited protein production. If the ribonucleotide diphosphates are reduced by ribonucleotide reductase into a deoxy-diphosphates, they will be incorporated into DNA. 5-aza-dC is a more potent demethylating agent than 5-azaCR, because it is only incorporated into DNA. Normally, when a cytosine is methylated by DNMTs, the DNMTs flip out the cytosines from the double helix to form an intermediate complex in which S-adenosylmethionine (Ado-met: the methyl group donor) is incorporated. This allows methyl group transfer from Ado-Met

to the C5 of cytosine and the release of the enzyme in a β -elimination reaction. On the other hand, if a cytosine analog is incorporated into DNA a complex is formed but the nitrogen atom in the position 5 of the ring prevents the release of the enzyme and results in the formation of a covalent complex. Thus DNMTs no longer methylate DNA, and subsequent DNA replication cycles will lead to a progressive demethylation (Figure 1.5.3.1).

As I described in the first chapter of this introduction, DNMT1 interacts with a number of proteins linking the enzyme to the nuclear matrix and targeting it to replication foci and repair sites. In addition, it contains sites for binding of HDACs and other proteins involved in transcriptional repression. 5-azaCR and 5-aza-dC can cause conformational changes in DNMT1, thus altering its interaction with other proteins; in particular, this could result in the inhibition of methylation and dissociation of HDACs, leading to rapid remodelling of chromatin and restoration of gene expression.

The mechanism of action of cytidine analogs has been studied for DNMT1. There have been no reports describing effects on the catalytic activity of DNMT3a or 3b, but given the conservation of the mechanism of cytosine DNA methylation, we could suppose that they are similar to DNMT1, which is the most abundant DNA methyltransferase in cancer cells (Christman, 2002).

5-azaCR and 5-aza-dC are highly toxic in cultured cells and animals. Indeed, the first therapeutic application of 5-azaCR was cytotoxic in the treatment of myelodysplastic syndromes and acute myeloid leukaemia. It is very difficult to distinguish whether the consequences of the treatment depend on the re-expression of particular genes, such as tumour suppressors, or on particular defects occurring in DNA, that set in motion the complex machinery of cellular responses to DNA damage. Studies performed on mutant cell lines expressing lower levels of DNMT1 demonstrated that these cells were substantially more resistant to the toxic effects of the demethylating drug 5-aza-dC than the heterozygous or wild type cells with normal DNA methylation levels. This could be

explained by the covalent binding of the enzyme to DNA, rather than the DNA methylation, being the key mediator of the drug induced cytotoxicity (Juttermann et al., 1994).

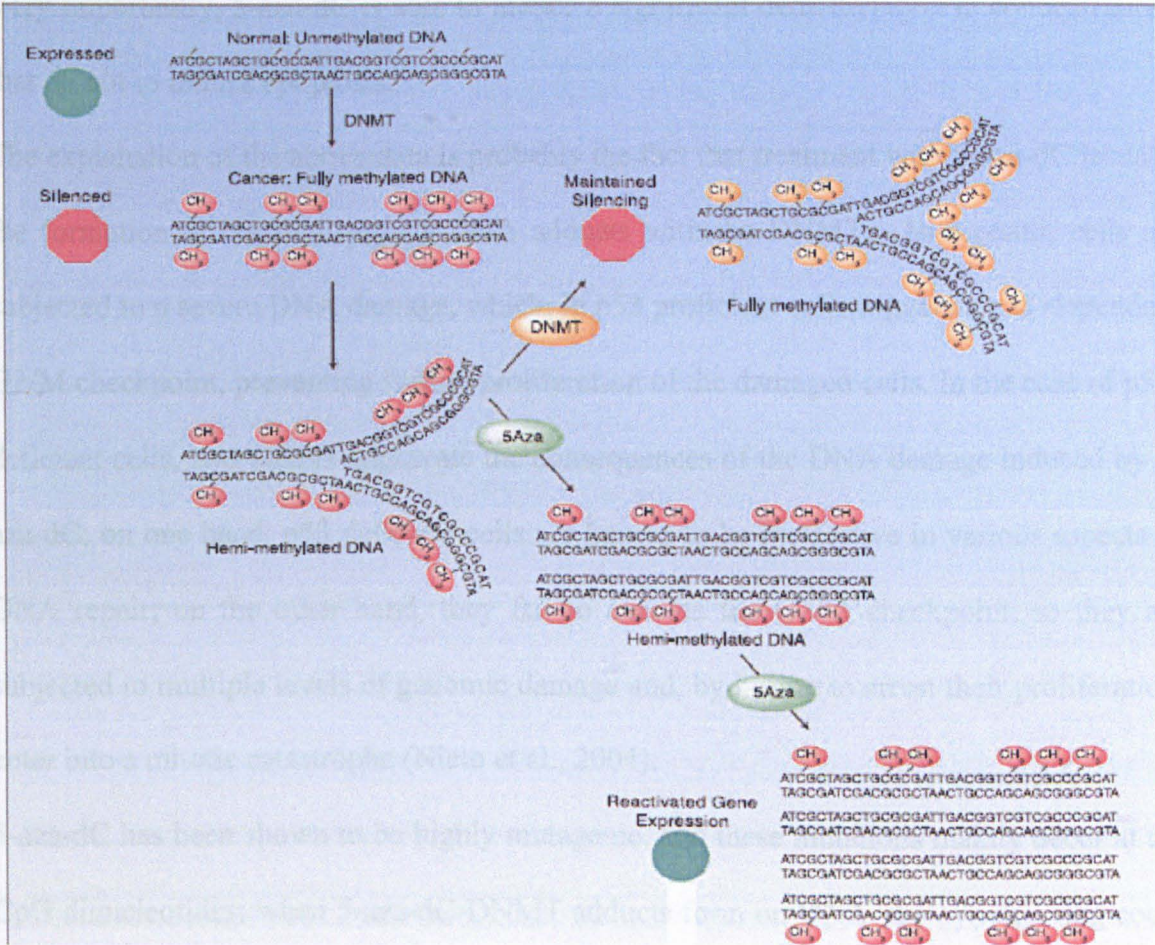


Figure 1.5.3.1 Kinetic of DNA demethylation induced by 5-aza-dC

From (Issa, 2007), modified

5-aza-dC has been shown to be very effective in inducing apoptosis in p53-null cells in a dose-related way. The relationship with the p53 deficiency is very high, but, if we consider its demethylating properties rather than the apoptotic ones, these are not dependent on p53 status.

Very importantly, 5-aza-dC is able to induce a significant demethylation at concentrations that do not induce apoptosis.

The explanation of the above data is probably the fact that treatment with 5-aza-dC leads to the formation of covalent protein-DNA adducts with the DNMTs. As a result, cells are subjected to a severe DNA damage, which, in p53 proficient cells triggers a p53-dependent G2/M checkpoint, preventing further proliferation of the damaged cells. In the case of p53-deficient cells, two factors aggravate the consequences of the DNA damage induced by 5-aza-dC: on one hand, p53 deficient cells are known to be ineffective in various aspects of DNA repair; on the other hand, they fail to execute the G2/M checkpoint, so they are subjected to multiple levels of genomic damage and, by failing to arrest their proliferation, enter into a mitotic catastrophe (Nieto et al., 2004).

5-aza-dC has been shown to be highly mutagenic, and these mutations mainly occur at the CpG dinucleotides; when 5-aza-dC-DNMT adducts form on DNA, the cytidine ring could open through the disruption of the N1-C6 bond and the subsequent hydrolysis, leading to the inactivation of the enzyme and to dangerous breaks on DNA. The opened ring represents a pre-mutagenic lesion that can induce C:G→G:C transversions, but also C:G→T:A transitions and C:G→A:T transversions (Jackson-Grusby et al., 1997).

In order to overcome the toxicity related to the covalent complexes of DNMT/5-aza-dC on DNA, new schedules of treatment have been adopted, by which the demethylating properties could prevail over toxicity. To this aim, *in vitro* experiments have shown that optimal methylation inhibition occurred at low doses (Nieto et al., 2004). These data have been further confirmed by clinical trials, showing that low-dose exposures leads to greater responses, also in terms of DNA methylation, and are associated with less toxicity

(Appleton et al., 2007; Issa et al., 2004). This is a very important concept, because this therapeutic approach is more feasible for combination schedule of treatment, above all in solid tumours, where the demethylating drugs alone do not seem to be successful to date.

However, combination therapy also allows overcoming another limit of the cytidine analog treatment, that is the rapid DNA re-methylation within few days after stopping demethylating drug administration.

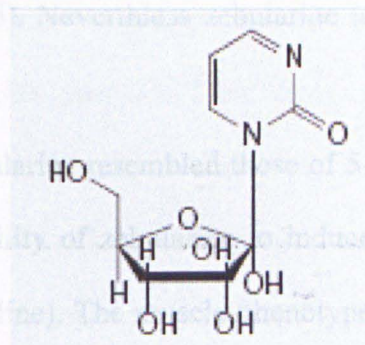
The two DNA methyltransferase inhibitors, azacytidine and decitabine represent the most active agents for the treatment of myelodysplastic syndrome (MDS) patients. The therapy induces complete responses, lasting months to years in some patients, even though resistance seems to develop in the majority of them with an unknown mechanism (Goffin and Eisenhauer, 2002). These drugs have recently obtained the FDA approval in this kind of disease.

Among the other azacytidine derivatives, we have to consider the dihydro-5-azacytidine (DHAC) and the 5-F-CdR. DHAC is hydrolytically more stable and less cytotoxic than 5-aza-CR and it has been shown to inhibit methylation in human lymphoid and leukaemia cell lines, as well as in tumour bearing mice.

5-F-CdR has a complicated metabolic activity in mammalian cells and this could prevent its further development in clinical trials.

1.5.3.2 Zebularine

A disadvantage of the azanucleosides is their instability in aqueous solutions, which could complicate their use in the clinical setting. Zebularine is cytidine analogue containing a 2-(1H) -pyrimidinone ring (Driscoll et al., 1991) that was originally developed as a cytidine deaminase inhibitor because it lacks an amino group at position 4 of the pyrimidine ring.



In addition, unlike azanucleosides, zebularine is stable in aqueous solution up to a pH of 12. It retains similarities to mechanism of DNMT inhibition of 5-aza-dC (see above), but its chemical characteristics result in differences in potency as a demethylating agent. When zebularine is inserted into DNA as cytidine analog, it becomes the target for DNMT1 enzymatic activity: during methylation reaction, the targeted zebularine is rotated out of the helix into the catalytic pocket of the enzyme, (while the dG on the complementary strand remains stacked within the DNA helix), and it determines the covalent attachment of the enzyme to DNA, which will be in turn depleted from the cellular pool and preventing its methylating activity.

The main chemical difference with 5-aza-dC, besides the loss of -NH₂ in position 4, is that zebularine lacks nitrogen in position 5, so why is the bond with DNMT permanent? And why does β elimination not occur? The reason is that the C5 of zebularine is not active, because the C6 chemistry resembles that of a cytidine deaminase inhibitor, rather than that of a DNMT inhibitor; therefore reaction with AdoMet does not take place (Zhou et al., 2002). Before being incorporated into DNA (at the GCGC target site), zebularine needs to be first phosphorylated and then converted to a deoxynucleotide: this represents the rate-limiting step of the reaction. However its ribose moiety determines its incorporation into RNA as well as into DNA. This characteristic, together with its peculiarity as cytidine deaminase inhibitor, probably lowers the effective availability of zebularine as a demethylating agent. Unfortunately a more direct route as deoxyribonucleotide is not currently available, since 2'-deoxyzebularine is not active, probably because it is not phosphorylated (Marquez et al., 2005; Ben Kasus et al., 2005). Nevertheless zebularine is able to induce and to sustain DNA demethylation over time.

The first studies showing the demethylating properties of zebularine resembled those of 5-aza-CR in the early 1980s by Jones et al., that is, the capability of zebularine to induce muscle cell formation in 10T1/2 cells (mouse embryonic cell line). The muscle phenotype

has been reported to be induced by inhibitors of cytosine methylation, suggesting that zebularine inhibits DNA methylation in these cells.

Undoubtedly one of the most valuable characteristic of zebularine is its stability in both acid and neutral solutions. This allows its administration via the oral route and, together with minimal *in vivo* toxicity, this makes it a promising clinical candidate for reversing DNA methylation and to be used as drug for cancer chemotherapy, chemoprevention and, in general, epigenetic therapy.

Demethylating properties of zebularine have been further appreciated because it has also been shown to be more specific for cancer cells thus reducing toxic effects in normal cells. This advantageous effect seems to depend on differential metabolism between cancer and normal cells, mainly related to lower levels of the uridine/cytidine kinase in normal cells compared to cancer cells. This could account for a decreased zebularine incorporation into both RNA and DNA of normal fibroblasts (Cheng et al., 2004b). However, cancer cells exhibited a greater growth inhibition, probably as a direct result of the incorporation of the drug into DNA. At this purpose zebularine, at the concentrations inducing DNA demethylation, results to be effective in inducing apoptosis in p53-deficient cells (usually tumour cells), even though this effect is not as marked as in the case of 5-aza-dC (it is roughly 50% of that of 5-aza-dC). The demethylating properties of zebularine are further supported by the depletion of DNMT enzymes following treatment: cancer cells show a complete depletion of DNMT1 and partial depletion of DNMT3a and DNMT3b2/3. This apparent specificity of the 2-(1H)-pyrimidinone ring for DNMT1 is unexpected, since all known DNMTs appear to utilize the same mechanism of action. Perhaps zebularine exhibits a greater enzymatic binding affinity for DNMT1 than for other DNMTs (Cheng et al., 2004a); among them, DNMT3b has per se a reduced catalytic activity, while DNMT3a is expressed throughout the cell cycle, as opposed to DNMT1 and DNMT3b, which are cell cycle regulated. The depletion of DNMT3a can therefore only occur during the S phase, in which the 2-(1H)-pyrimidinone ring is incorporated into DNA. Following oral

administration, zebularine has been shown to reactivate the methylated p16 gene and to elicit tumour suppression of human T24 bladder cancer cells in mouse xenografts, without significant toxicity (Cheng et al., 2003).

Another appreciable characteristic of zebularine is its activity as a cytidine deaminase inhibitor. Cytidine deaminase is a key enzyme in the catabolism of 5-aza-dC, which is converted to 5-aza-2'deoxyuridine, a completely inactive product. This represents a potential obstacle for curative therapy with 5-aza-dC, mainly because of the very high levels of cytidine deaminase in human liver and spleen. Moreover, resistance to 5-aza-dC can be mediated by increased levels of cytidine deaminase in tumours. In contrast, zebularine is not degraded by this enzyme, even though it is characterized by a longer metabolic process before being inserted into DNA.

All these factors support firstly the practicability of zebularine as demethylating agent *in vivo* and then the possible use of a combination of zebularine with 5-aza-dC. Actually this kind of approach has just been tested in the murine leukaemia cell line L1210, where it produced a greater inhibition than either of the two agents administrated alone (Lemaire et al., 2005).

1.5.3.3 Non nucleoside analog inhibitors of methylation

The limitations of the cytidine analogs, mainly related to toxicity both *in vitro* and *in vivo*, have prompted investigators to explore new strategies that could revert aberrant methylation in cancer cells. Actually, the data obtained up to now by testing non-nucleoside analogs as DNA methylation inhibitors, are still controversial and the re-activation of epigenetically silenced genes likely depends on the particular locus and the cell line being considered.

Procainamide was approved by FDA as a specific agent for the treatment of cardiac arrhythmias and only later were its demethylating properties discovered (Lin et al., 2001a; Segura-Pacheco et al., 2003). Procainamide is able to bind CG-rich DNA sequences and

this has supported the idea that it might be a demethylating agent. However it has also been shown to specifically target DNMT1. In fact procainamide failed to reduce genomic ³H-5dC content in HCT116 colon cancer cells where DNMT1 was genetically deleted, but it significantly reduced global methylation in parental HCT cells and HCT cells with no expression of DNMT3b. This demonstrated that de novo methyltransferases were indeed not sensitive to procainamide inhibition. During methylation of the hemimethylated strands of the DNA, procainamide acts by reducing the affinity of DNMT1 for both DNA and AdoMet (methyl donor) and, as a consequence, the DNMT1 processive mechanism of action is compromised. However procainamide is a partial inhibitor of DNMT1 and the velocity of the reaction does not reach zero even at very high concentrations (Lee et al., 2005).

Procainamide has a good safety profile with none of the major disadvantages of nucleoside analogs, and although only a few genes and cell lines have been analyzed, it seems to reactivate genes silenced by methylation.

Procaine (PCA) is a drug approved by the FDA for use as a local anesthetic. Its ability to bind CpG-rich DNA has suggested that it could also be a demethylating agent. This has been supported by experimental data, showing a global genomic methylation decrease after treatment of the MCF-7 breast cancer cell line with increasing concentration of PCA and the demethylation and re-expression of a CpG island associated gene (RAR β 2) (Villar-Garea et al., 2003). PCA increased the antitumour activity of several conventional anticancer drugs, such as cisplatin, mitomycin C, doxorubicin, but how this property could be related to the hypomethylation is a matter of debate and, up to now, no relationship between the re-expression of a particular gene by PCA and sensitization to chemotherapy has been demonstrated.

Other strategies to revert aberrant methylation in cancer have been tested, but they still are at preclinical level of evaluation. Among them is methioninase, which depletes methionine (the methyl donor in methylation reaction) (Machover et al., 2002) and (-)-

epigallocatechin-3-gallate (EGCG, green tea extract), whose demethylating properties were demonstrated in a human esophageal cancer cell line, where it reverted silencing of p16, RAR β , MGMT and hMLH1 genes. A suggestion of its possible involvement in demethylation comes from molecular modelling studies showing that EGCG is well accommodated in a hydrophilic pocket of DNMT1 (Fang et al., 2003).

Other examples of demethylating agents include arsenic and phenethyl isothiocyanate: the latter, in particular, has been used to reactivate GSTP1 expression. The lack of further preclinical and clinical development does not corroborate, at the moment, the potential of these drugs as clinical demethylating molecules (Wang et al., 2007).

To overcome the limits of the classic molecules used until now, rational design studies of the target to be silenced have helped the synthesis of new molecules, which, hopefully, could have activity both *in vitro* and *in vivo*.

RG 108 is the first DNMT inhibitor produced by a rational drug design: it was selected for its ability to block the active site of the human DNMT1 enzyme in an *in silico* model. Experiments performed *in vitro* confirmed that RG108 was able to reactivate several epigenetically silenced tumour suppressor genes. At the same time, the compound did not seem to alter the methylation status of centromeric repeats and this implies that RG108 treatment should not affect chromosome stability (Brueckner et al., 2005).

S110, a 5'-aza-pG-3-dinucleotide contains 5-aza-dC and operates via similar mechanism of action to the parent compound to inhibit DNA methylation, induce expression of the p16 tumour suppressor gene and inhibit tumour cell growth, but it is resistant to deamination by cytidine deaminase, which rapidly depleted the plasma levels of 5-aza-CdR (Yoo et al., 2007).

In summary, the data available until now suggest that, despite their considerable cytotoxicity, azanucleoside inhibitors show the strongest demethylation effects (Chuang et al., 2005). The epigenetic changes induced by non-nucleoside inhibitors seem to be more controversial and their action could be gene-specific or cell line-specific; however the

concentrations used in experimental conditions showing demethylation were very high and this could make their application in clinical trials very difficult (Stresemann et al., 2006).

1.5.3.4 HDAC inhibitors

Among the modifications affecting histone proteins, acetylation/deacetylation is the most feasible target for an epigenetic therapy in cancer cells, because histone acetylation is only related to transcriptional activation and deacetylation is only related to transcriptional silencing. As a consequence, treatment with molecules that specifically inhibit histone deacetylation could lead to re-expression of genes that are important in tumour suppression.

In the last few years, many molecules with this specific target have been synthesized and tested in different cellular systems, in preclinical studies and in phase I/II of clinical trials, even though many concepts need to be clarified about their mechanism of action.

Although histone deacetylation has a general and fundamental role in regulating gene expression, HDACi seem to directly affect transcription of only a relative small number of genes. Recent analysis using microarrays estimate that about 10% of genes are affected by this kind of treatment. The reason why this happens is not clear, but we have to consider that histone tails are targets of many other covalent modifications that can also affect gene expression. In particular, histone methylation, which proceeds after deacetylation of histone tails, can induce heterochromatin and euchromatin gene silencing. In these instances, addition of HDACi would not be sufficient to reactivate the silenced genes.

A relatively wide range of compounds have been identified that are able to inhibit the activity of class 1, class 2 and class 4 HDACs. They derive from both natural sources (depsipeptide, apicidin, trichostatin A, sodium butyrate) and from synthetic routes (for example, hydroxamic acid derivatives). Crystallographic studies using trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) indicate that these compounds inhibit HDAC activity by interacting with their catalytic site, thereby blocking substrate access.

These inhibitors target, with more or less the same efficiency, every class of HDACs. Only a few molecules are emerging as preferential inhibitors of class 1 versus class 2 HDACs, and even fewer (that is tubacin for HDAC 6) are able to discriminate efficiently among HDACs that belong to the same class. If we consider HDACi as molecules inducing gene expression, different HDACi activate a common set of genes, and this indicates that that certain loci are more susceptible to these compounds than others.

HDACi show specificity for tumour cells rather than normal cells, and this makes them, despite their quite unknown effects on cellular pathways, appropriate molecules for clinical development.

The research developed until now on these molecules have highlighted that their efficiency is rarely associated to the re-expression of a particular gene, such as tumour suppressors in cancer; their anticancer potential stems from their ability to affect several cellular processes that are deregulated in neoplastic cells: cell-cycle arrest, differentiation, apoptosis and, in addition, activation of host immune response and inhibition of angiogenesis. So HDACi could also work through non-transcriptional mechanisms, and, as a consequence, they might be considered as partial epigenetic drugs. In leukemias, HDACi have demonstrated differentiating properties, inhibiting cell cycle and blocking proliferations of cancer cells. It also been shown that HDACi-induced growth arrest is tightly linked to the induction of the cdk inhibitor p21, and in some cases this arrest seems to be irreversible and to resemble the phenomenon of replicative senescence (Minucci and Pelicci, 2006).

HDACi are also involved in apoptosis, by the upregulation of the pro-apoptotic BCL2 family members and the downregulation of the anti-apoptotic proteins of the same family; this is associated with the subsequent activation of the caspases.

As regard the use of HDACi in cancer therapy, they have shown encouraging results in preclinical models, not only in leukemias, but also in solid tumours. Specifically, butyrates inhibited the growth of colon, prostate, endometrial and cervical carcinomas; valproic acid

(already used for epilepsies) delays the growth of primary breast cancer and inhibits the size and number of metastasis found in the lung.

In general, SAHA, CBHA and TSA markedly suppressed the growth of a range of solid tumours xenografts, and inhibited the progression of spontaneous tumours that were induced with carcinogens. Their therapeutic effect is usually reached at very high dosage (range of concentrations is millimolar); however they exhibit a short half life and are able to overcome the multidrug resistance that is mediated by overexpression of P-glycoprotein. Phase I/II of clinical trials have begun, with HDACi administered both as single agents and in combination. Generally they have shown low toxicity, which might be at least in part the result of the non-specific side-effects of each individual HDACi, rather than the consequences of inhibiting HDAC *per se* (Johnstone, 2002).

If we strictly consider HDACi as molecules reverting epigenetic modification of the chromatin, the most logical therapeutic consequence is to combine them with demethylating agents, because methylation and histone deacetylation contribute each other to set of a closed conformation of the chromatin. Functional synergy between DNA methylation and histone deacetylation has been shown experimentally using 5-aza-dC and TSA. TSA alone had no effect on transcription of epigenetically silenced genes and 5-aza-dC alone weakly induced transcription. However, the combination of both 5-aza-dC and TSA resulted in a robust induction of gene expression, which indicates that, although DNA methylation and histone deacetylation act in synergy to silence genes, DNA methylation is the dominant mechanism in silencing (Cameron et al., 1999).

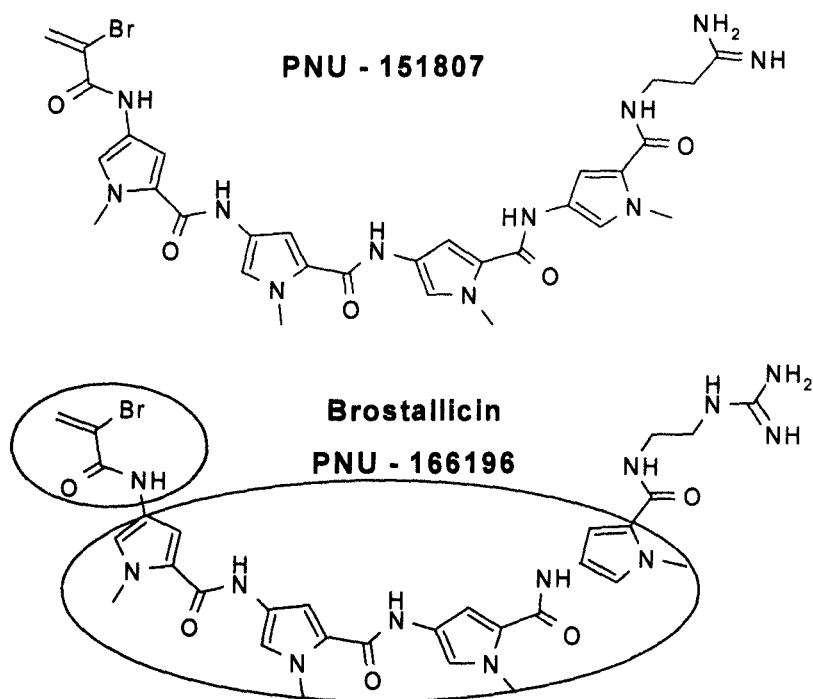
1.5.4 BROSTALLICIN

Brostallicin (PNU 166196) is a synthetic α -bromoacrylic derivative of distamycin A, belonging to the DNA Minor Groove Binder (MGB) class of anticancer drugs. Like distamycin A, it is characterized by a four-unit pyrrol carbamoyl backbone (fig.1.5.4,A), which enables brostallicin to bind to AT regions present in the minor groove of the DNA. One of the main derivatives of distamycin A with anticancer properties has been tallimustine, whose excellent antitumour activity in preclinical studies was compromised by severe myelotoxicity in clinical trials, preventing the administration of potentially therapeutic doses (D'Incalci and Sessa, 1997). While tallimustine is a nitrogen mustard derivative, brostallicin belongs to the α -halogenoacrylic derivatives of distamycin A, in which the halogen-group is represented by bromo. Among these different halogen containing molecules synthesized, α -bromo and α -chloroacrylamido-derivatives showed a significant cytotoxicity, while the α -fluoroacrylamido and acrylamido derivatives were inactive.

Among the bromoacrylic MGBs, PNU 151807 (fig.1.5.4,A) was initially considered because of its *in vitro* cytotoxicity and *in vivo* antitumour activity significantly higher than that of tallimustine. PNU -151807 was able to non-covalently bind TA rich-regions in the minor groove of DNA, but was unable to alkylate DNA in classical *in vitro* assays (Broggini et al., 2004). Its α -bromoacrylic moiety seems to interfere with cell cycle progression by so far unknown mechanism.

Brostallicin *per se* did not produce any alkylation in any of the selected DNA interacting regions; this capability was acquired only in the presence of cellular thiols (particularly GSH), whose intracellular concentration is in the millimolar range. These chemical species allow brostallicin to perform a first-step Michael-type attack, followed by a further reaction leading to a second nucleophilic substitution, resulting in turn in alkylation of DNA nucleophilic sites (fig.1.5.4,B). The fundamental need of nucleophilic species

(A)



(B)

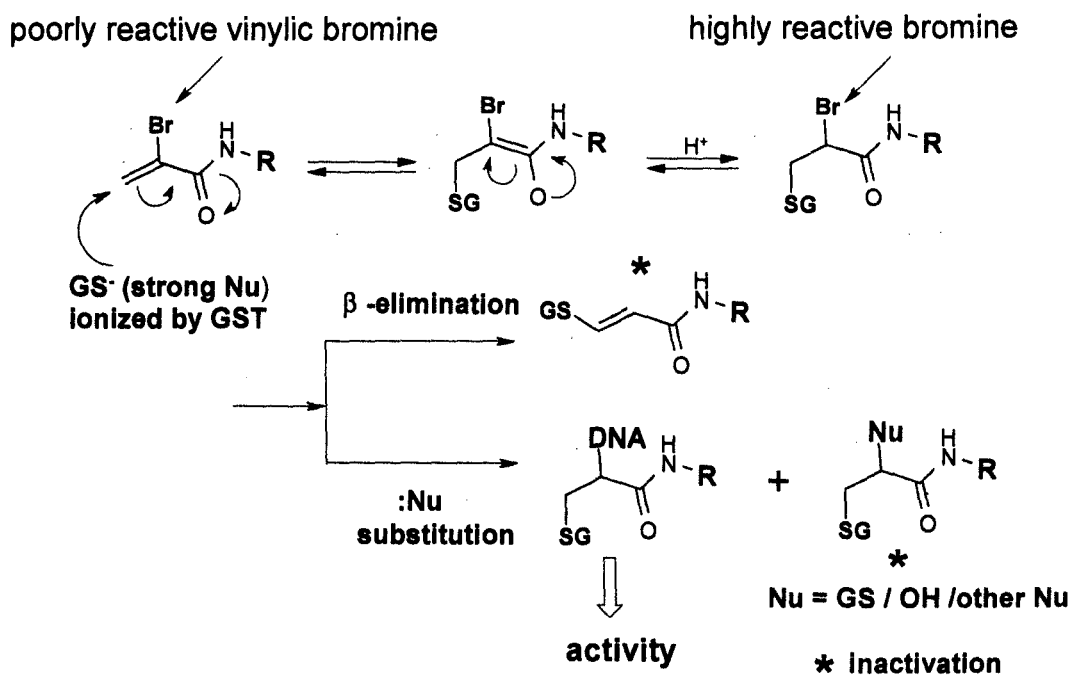


Figure 1.5.4 Brostallicin structure and reaction

(A) Chemical structures of PNU 151807 and brostallicin. The α -bromoacrylic group (blue) and the four-unit carbamoyl backbone (red) are highlighted on brostallicin formula.

(B) Role of the GSH/GST system in the interaction between DNA and the α -bromoacrylic derivatives

for the antitumour activity of brostallicin was demonstrated by experimental data showing that brostallicin had an increased activity against melphalan-resistant L1210 cells (L1210/L-PAM), which are characterized by high levels of glutathione, compared to wild type L1210 cells (Broggini et al., 2004).

As I described previously high levels of the GSH/GST system restricts the cytotoxicity of a substantial number of anticancer agents and, as a consequence, it usually represents a mechanism of resistance. In marked contrast to this, in the presence of GSH/GST, brostallicin is able to bind covalently to AT-rich regions in DNA, and with a sequence specificity that is different to that of its precursor, talimustine.

1.5.4.1 In vitro and in vivo brostallicin antitumour activity

In vitro and *in vivo* studies strengthen the concept that the antitumour activity of brostallicin is increased in tumours with high GSH/GST levels. Among the different GST classes, GST π and GST μ are more effective than the α -class in catalysing the reaction between brostallicin and GSH. This might be important clinically, because GST π is the most prevalent GST class expressed in tumours, and brostallicin could exert its antitumour activity if those tumours become resistant to other drugs by its upregulation.

When human GST π cDNA was transfected into the human ovarian cancer cell line A2780, a 2- to 3- fold increase in the intracellular GST π levels resulted in a 2- to 3- fold increase in the cytotoxic activity of brostallicin. These results were further confirmed in the human breast carcinoma cell line, MCF-7, transfected with GST π : here brostallicin showed a 5.8 fold increase in cytotoxicity in the transfected cells versus control cells (Geroni et al., 2002).

The *in vitro* data were further supported by *in vivo* experimentation using A2780 control (wild type, wt) and transfected (A2780/GST) cells transplanted into immunodeficient mice. The antitumour activity of brostallicin was clearly increased in tumours overexpressing GST π . In contrast no changes in cisplatin efficacy were observed. This increased tumour

antitumour activity of brostallicin was not associated with an increased toxicity in mice (Geroni et al., 2002).

Another appreciable characteristic of brostallicin is the maintenance of its cytotoxicity in cells with defects in DNA mismatch repair system (MMR) (Fedier et al., 2003). MMR proteins recognize mismatched base pairs in the DNA, arisen either spontaneously during metabolism or from modified nucleotides provoked by physical and chemical agents, and are thought to link DNA damage recognition to an apoptotic pathway, thereby preventing mutagenesis, tumourigenesis and tumour progression. Tumours resulting from MMR-deficiency include the hereditary non polyposis colon cancer and some sporadic carcinomas such as mammary, ovarian or endometrial cancer. However loss of MMR results in resistance to a variety of widely used anticancer drugs, including the topoisomerase I poisons camptothecin and topotecan, the topoisomerase II poisons doxorubicin, epirubicin, mitoxantrone and etoposide and some platinum compounds such as cisplatin and carboplatin.

Among the MGB agents, MMR status can affect the antitumour activity of tallimustine, but, interestingly, this is not the case for brostallicin. Probably the covalent reaction product of brostallicin with DNA is not a substrate for MMR, whereas tallimustine-alkylated DNA by is recognized by MMR (Fedier et al., 2003).

In this scenario, brostallicin might be a good candidate for treatment of those tumours showing resistance to cisplatin either through deficiencies in MMR or increased levels of the GSH/GST system, or, indeed, both.

1.5.4.2 Brostallicin Combinations

The above characteristics make brostallicin a good candidate for combination therapy with different anticancer agents that can influence the intracellular GSH/GST levels. The combined use of anticancer agents is a common strategy in clinical chemotherapy, especially in metastatic cancer (Ten Tije et al., 2003; Leahy et al., 2007); thus a good

rationale for combinations represents a favourable mark for the development of new molecules.

The demonstration that GST π enzymatic activity increases in tumour cells in response to cDDP, DX and CPT-11 treatment, prompted investigators to explore the *in vivo* combination of cDDP and brostallicin. The results supported the starting hypothesis, because cDDP administrated before brostallicin clearly had a synergistic antitumour activity, compared to the opposite schedule (brostallicin before cDDP), in which no increased antitumour activity of brostallicin was demonstrated. These results have been confirmed using other combinations, including, besides brostallicin, DX, CPT-11, and taxotere, but again only when these drugs precede brostallicin. Interestingly from a therapeutic point of view, the increased antitumour activity was not associated with an increased toxicity, especially because the use of a well designed drug combination allows lowering the concentrations of the single agents involved (Sabatino et al., 2003).

An additional attractive therapeutic application is presented by tumours with low GST levels due to hypermethylation of the GST gene promoter. This is particularly relevant for prostate carcinoma, where more than 90% of tumours are characterized by this epigenetic modification, responsible for GST expression silencing.

The combination of brostallicin with agents able to revert hypermethylation and allow the re-expression of GST π gene could therefore result in a tumour-selective synergistic activity.

1.6 PERSPECTIVES ON THE COMBINATIONS OF DEMETHYLATING MOLECULES WITH CYTOTOXIC DRUGS

Tumour treatment with demethylating agents is becoming more and more appreciable as new genes are being discovered to be silenced in an “epigenetic way”. For this reason the search for molecules able to inhibit the mechanism of the epigenetic silencing has been strengthened in the last years, but, at the moment, the results obtained with the non nucleoside analogs or with rationally designed inhibitors are not as strong as those obtained with the “oldest” demethylating nucleoside analog 5-aza-dC. Zebularine has demonstrated to be a promising candidate in this kind of therapy and we need to more deeply study its demethylating effects on a broader spectrum of genes. What is clear is that drugs targeting methylation are more effective on blood tumours than in solid ones. In fact data obtained by administrating demethylating agents on solid tumours (actually very few if compared to studies on blood) demonstrated not a univoque outcome, especially when they are administrated as single drug. Moreover there is an increasing interest in the treatment of these tumours with combinations of epigenetic drugs and the classic cytotoxics, which are currently used in chemotherapy. The rationale for these combinations is further corroborated by the knowledge that tumours might epigenetically silence specific genes as mechanism of resistance to therapy, as it has been well demonstrated for hMLH1 in ovarian cancers after treatment with cisplatin. As a consequence, treatment with molecules reverting (hopefully: we have to remember that there is no specificity for a particular gene) the silenced target could re-sensitize tumours to chemotherapy; in fact, *in vivo* studies, showed that combination of 5-aza-dC with cisplatin, carboplatin and temozolomide on A2780 with methylated hMLH1 (A2780/cp70) resulted in an increased antitumour activity compared to the drugs administrated alone, because of the re-expression of hMLH1 after treatment with 5-aza-dC (Plumb et al., 2000). New studies are

exploring the feasibility of zebularine in these combinations, because treatment with 5-aza-dC often determines hematologic toxicity (Balch et al., 2005).

I have described before that therapy with demethylating agents could allow the re-expression of genes, which have become methylated during the process of tumour transformation, thus resulting in growth inhibition. This has been demonstrated for tumour suppressors, like p16 in bladder cancer, whose re-expression determined the growth inhibition of tumours *in vivo*.

But it is remarkable that methylation affects not only tumour suppressors, but also other genes, whose proteins could have an important role in the treatment regimens, like GSTP1 in prostate cancers.

Thus the characterization of the epigenome of each kind of tumour could help to more specifically design drug combinations, with the result of a more effective response to therapy.

2. AIMS

The mechanism of action of the minor groove binder brostallicin has been extensively studied in our laboratory for many years. Previously I have demonstrated that this new anticancer drug can be successfully used in combination with cytotoxic drugs that are able to induce the overexpression of the GSTpi enzyme as a mechanism of resistance (Sabatino et al., 2003). In order to widen the spectrum of brostallicin activity, new combination strategies have been considered and in this thesis I explore the combination with epigenetic therapies, specifically, demethylating agents. Previous studies that have focused on the epigenetic modifications of human cancer have shown that prostate cancer is characterized by an extensive methylation of the GSTP1 promoter.

For this reason the overall aim of this study has been to combine a demethylating drug with brostallicin in a cellular model of prostate cancer (LNCaP cells) which has methylated GSTP1, in order to widen the antitumour activity of this new anticancer drug to a tumour model with an epigenetically silenced GSTpi enzyme.

This general objective was pursued through the following steps:

1. firstly, I have verified that LNCaP cells can be sensitized to brostallicin, by transfecting them with a GSTP1 expressing plasmid construct;
2. I have assessed several demethylating drug for their ability to increase the cytotoxic activity of brostallicin in LNCaP cells through *in vitro* combinations; this step has allowed me to test several schedules of combinations with different molecules known to be able to revert DNA methylation;
3. I have translated the most suitable combination found *in vitro* to an *in vivo* system, in particular to a xenograft model;
4. I have analyzed GSTP1 expression and GSTP1 promoter methylation after treatment with the selected demethylating agent;
5. I have analyzed other genes that might be affected by the demethylating agent treatment, due the possible non-specific action of the drugs reverting epigenetically silenced genes.

These strategies are in line with new therapeutic approaches now in the clinical phases of development, especially for solid tumours.

In the last part of my project, I have evaluated the effects of decitabine on MAGE1A methylation levels in normal and tumour samples of patients recruited into a phase I clinical trial. The specific aim of this part of the project was to verify that indeed, in the clinical setting, demethylating agents can significantly re-induce the expression of silenced genes in solid tumours.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

Cell culture procedures were carried out aseptically in class II humidified laminar flow hoods. Cells were maintained in a Heraeus Auto-Zero CO₂ incubator at 37°C with 5% CO₂ in air and tested for mycoplasma contamination once a month by PCR (Forward primer: 5'-TGCACCATCTGTCACTCTGTAAACCTC; Reverse primer: 5'-ACTCCTACGGGAG GCAGCAGTA).

The human cancer cell lines used in my investigations were: the human prostatic cancer cell lines LNCaP and Du145 and the human breast cancer cell line, MCF-7.

LNCaP, Du145 and MCF-7wt cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA); MCF-7/neo and the sublines over-expressing GST isoforms, MCF-7/GST π , MCF-7/GST α , MCF-7/GST μ , were kindly supplied by Dr. Jeffrey A. Moscow (Moscow et al., 1989).

3.1.1 Culture Conditions

The LNCaP and Du145 cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS, Sigma; for Du145 cell culture, FCS was previously inactivated at 56°C for 30 minutes) and 2mM L-glutamine (BioWhittaker). The LNCaP/GST π subline was grown in the same conditions with the medium supplemented with G418 500 μ g/ml (Invitrogen (Milan,Italy): 100X stock solution, 80mg/ml, in 100mM Hepes pH 7.4 is stored at -20°C until use). The MCF-7 cell line was maintained in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% FCS and 2mM L-glutamine and the MCF-7 sublines, over-expressing the different GST isoforms, were grown in D-MEM medium, supplemented with 10% FCS, 2mM L-glutamine and G418 antibiotic, as reported (Moscow et al., 1989). Media were purchased from Sigma and contained supplements necessary for the growth of the cells except for the serum (stored at 4°C as 50ml aliquots, when thawed), which was added when the media were prepared before use.

Cells were passaged twice a week before they reached confluence, to maintain a logarithmic growth. The cells were replaced with fresh liquid nitrogen stocks after every four to five months of culture. Procedures to detach and subculture were the same for all the cell lines used and consisted of two washes with warm sterile phosphate buffer saline (PBS, Sigma) and detachment with a ready-to-use solution of trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA, Sigma). The trypsin activity was stopped by adding FCS-containing medium.

After centrifugation at 1200rpm for 10 minutes at room temperature, cells were resuspended in the appropriate medium, counted (Coulter Counter, ZM) and seeded at the required density (LNCaP cells: 10×10^4 cells/ml; Du145 and MCF-7 cells: 5×10^4 cells/ml).

3.1.2 Long term storage of cells

To generate and maintain batches of cells, exponentially growing cells were washed twice with PBS, harvested by trypsinization and centrifuged at 1200 rpm for 10 minutes at room temperature. The cell pellet was resuspended in culture medium containing 50% v/v of cryoprotective agent (Bio-Whittaker, Milan-Italy) and 20% FCS to a density of 5×10^6 cells/ml. Aliquots of 1ml of the cell suspension were kept on ice for 30 minutes, cooled slowly for 3 hours in liquid nitrogen vapour and then immersed in liquid nitrogen. Cells were recovered from the cell bank by rapid thawing to 37°C in a water bath, centrifuged at 1200 rpm for 10 minutes at room temperature, resuspended in 5ml culture medium and transferred to a T-25 tissue culture flask; one day later, the medium was removed and new, fresh medium was added.

3.1.3 Cell transfection and clone selection

To obtain LNCaP cells over-expressing GSTpi protein, LNCaP cells were transfected with GSTP1/pcDNA3 plasmid construct, which was kindly provided by Dr. Sergio Marchini (Department of Oncology, Mario Negri Institute), using aseptic conditions.

JM109 bacterial cells (stored at -80°C) containing the construct were scraped from the 2ml Nalgene tube and suspended in 10ml LB medium (1%w/v bacto-tryptone, Sigma, 0.5%w/v bacto-yeast extract, Sigma, 1%w/v NaCl, Sigma, to a final volume of 1l with deionised water. The medium was autoclaved for 15 min and ampicillin was added once it had cooled to 55°C) containing $50\mu\text{g/ml}$ ampicillin (LB-amp), in a sterile 50ml tube, and allowed to grow at 37°C in a shaking incubator at 30rpm. After 8h, 1ml was mixed with 100ml of a fresh solution of LB-amp in a 250ml conical flask, and bacterial cells were left to grow overnight at 37°C and 30rpm. Bacterial cells were then collected by a 30min centrifugation at 1700rpm at 4°C . After removal of LB medium, plasmid DNA was purified using a Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. The DNA recovered from the midi preparation was quantified using a spectrophotometer, by reading the absorbance at 260nm and 280nm. The quality of DNA prepared was determined by the ratio between 260nm and 280nm absorbance (ratio ≥ 1.8 for good quality samples). The amount of DNA was calculated considering that a solution of DNA $50\mu\text{g/ml}$ would give an absorbance of 1.0 at 260nm.

To transfect DNA into LNCaP cells, Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions. Briefly, cells were seeded at high density in a T-25 flask one day before transfection in order to have a 90-95% confluence at the time of transfection. Both DNA and Lipofectamine were diluted separately in Opti-MEM I Reduced Serum Medium (Invitrogen). After 5 minutes of incubation of Lipofectamine at room temperature, the diluted DNA was added (the final ratio was DNA $1\mu\text{g}$ to Lipofectamine 2000 $3\mu\text{l}$) and incubated for 20 minutes at room temperature. The mixture was then added to the cells (about $8\mu\text{g}$ DNA in a T-25 flask) which were incubated at 37°C until harvesting.

Two days later, the medium was removed and the cells detached with trypsin/EDTA solution. After counting, the cells were seeded in 15mm plates at density of 5000 cells/ml in RPMI medium containing the antibiotic G418 ($500\mu\text{g/ml}$).

A second transfection was performed using the same conditions, except the cells were selected with G418 at 1000 µg/ml.

At these antibiotic concentrations, parental cells are killed and the only cells able to grow are likely to be those which have integrated the transfected plasmid into their DNA. The plates were then kept at 37°C and the medium renewed every two-three days. Colonies were visualized under the microscope and single colonies isolated by using plastic cloning rings which were attached to the plate with sterile Vaseline. Trypsin/EDTA solution (20 µl) was introduced into the cloning rings and the detached cells were transferred to 6 well plates, containing 2ml of medium plus G418. Each clone was seeded in duplicate in two parallel plates: one plate was used to verify the presence of the gene of interest by western blotting and the other was used to maintain the clone for further studies and for long term storage.

3.1.4 Growth inhibition assays

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a very simple colorimetric method that allows the determination of the effect of drugs on cell growth. The assay is based on the ability of mitochondrial succinate dehydrogenase to metabolise the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into its formazan (Denizot and Lang, 1986; Mosmann, 1983). Briefly, 20µl of MTT (Sigma, 6mg/ml in sterile PBS) are added to the cells in 96 well plates and incubated for at least 4h at 37°C in the dark. The medium is then removed, and the insoluble formazan salts are dissolved with a solution of 0.025N HCl in isopropyl alcohol, and the optical density at 550 nm determined (spectrophotometer "Titertek", Dasit Milan-Italy).

To investigate the effects of drugs on cell growth, LNCaP, Du145 cells and MCF-7 cells were seeded in 96 wells plates and allowed to attach for at least 48h.

The cell concentration used for seeding depended on the cell line and the kind of drug treatment performed: when the effect of only a single drug was assessed, LNCaP cells (wild

type and the clones overexpressing GSTpi) were seeded at 7000 cells/well, while Du145 and MCF-7 cells were seeded at 3000 cells/well, due to different kinetics of cell growth (LNCaP cells have a doubling time of roughly 64h, while for Du145 and MCF-7 cells this is about 28-30h). When combination drug treatments (96h) were performed, LNCaP cells were seeded at 3500cells/well. For treatments longer than 96h (8 days, as described in Results 4.1.2.4), LNCaP and Du145 cells were pretreated in T-25 flasks, before being seeded in 96 wells plates prior to treatment with the second drug, which was added with a concentration range into sixtuplicate wells, for 72h; control untreated cells were incubated with an equivalent volume of fresh medium. Growth inhibition was calculated as percentage of the OD values obtained in drug free control cultures (%Ctrls), or, for the combinations, as percentage of the OD values obtained in the demethylating drug alone cultures. The mean concentration causing 50% growth inhibition compared to the cells (IC_{50}) was determined for each drug from at least three separate experiments and was calculated from the linear regression fitting of the growth inhibition curves.

For experiments involving pretreatment with 5-aza-dC (Results, fig. 4.1.3), LNCaP cells were seeded in 6 well plates at 20000 cells/well. They were allowed to attach for 48h, before adding 5-aza-dC 0.05 μ M. Because 5-aza-dC has a half-life of 12h at 37°C, medium was replaced daily with medium containing fresh 5-aza-dC. After two weeks, brostallicin at increasing concentrations was added for 72h. The effect of the combination of the two drugs was assessed by the crystal violet staining. 1ml of crystal violet (commercially available solution 1%w/v in water; BDH, Mila, Italy) was added in each well for 2 minutes at room temperature. After extensive washes in water, with the final two washes in distilled water, the plates were air dried.

The MTT assay was used to determine the toxicity of demethylating agents on LNCaP cells, by performing treatments at the same concentrations and exposure time used in the different combinations with brostallicin.

3.1.5 Statistical analysis

The effects of the drugs, alone or in combination, on cell viability were compared using the one-way Analysis of Variance (ANOVA, Statview Statistical Package SAS Institute Inc., third edition). Differences among the OD values obtained with the MTT assay were considered statistically significant when $p < 0.05$.

3.1.6 Drug solutions

Stock solutions were prepared according to the following table:

| <i>Drug</i> | <i>Stock solution concentrations</i> |
|---|---|
| 5-aza-dC (Sigma) | 4.4mM in PBS (stock solution stored at -20°C) |
| procaine (Sigma) | 3.67mM in PBS, freshly prepared |
| procainamide (Sigma) | 100mM in PBS, freshly prepared |
| zebularine (purchased by Nerviano Medical Sciences; Milan, Italy) | 4.4mM in PBS (stock solution stored at -20°C) |
| trichostatin A (Sigma) | 5mM in ethanol (stock solution stored at -20°C) |
| brostallicin (purchased by Nerviano Medical Sciences) | 1mg/ml in DMSO10% + PBS 90% freshly prepared |
| cDDP (Sigma) | 3.3mM in medium, freshly prepared (kept at 37°C for 30' before treatment) |

Table 3.1.1 Drug solutions

The stock solutions were diluted in culture medium to provide the appropriate concentrations for cell treatment.

3.2 DNA EXTRACTION

3.2.1 Genomic DNA extraction from xenograft tissue samples

Frozen tissue fragments, excised from LNCaP tumours of mice treated with zebularine (500mg/kg for two cycles, Results, fig. 4.2.2, A), weighing up to 50mg were used for DNA extraction on the MaxwellTM 16 Instrument (Promega), provided with the MaxwellTM DNA purification kit (Promega). This instrument purifies uses Magnesil® Paramagnetic Particles (PMPs), which provide a mobile solid phase that allows the capture, washing and elution of the target material. DNA was quantified using a Nano-Drop ND-1000 spectrophotometer, according to the manufacturer's manual (Celbio, Italy).

3.2.2 Genomic DNA extraction from human biopsies

Following written informed consent of patients involved in a phase I study of decitabine, receiving epirubicin, cisplatin and infusional 5-FU (ECF) for advanced gastric or oesophageal cancer, tissue samples were collected, on days +1 and +11 after decitabine administration, from primary tumour sites and normal mucosa of the patients. On the same days, blood samples were collected. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

Tissue samples (about 25mg) were ground in liquid nitrogen to a fine powder. DNA was extracted by using the reagents in the BACC2 Nucleon DNA extraction kit (Amersham Biosciences), after an overnight digestion at 50°C with the proteinase K solution (10µg/µl) of the kit. At the end of the extraction, DNA was precipitated by adding 2 volumes of cold absolute ethanol and then washed by adding cold 70% (v/v) ethanol. DNA was air dried and re-dissolved in an appropriate volume of nuclease-free water.

DNA was quantified using a Nano-Drop ND-1000 spectrophotometer, according to the user's manual.

3.2.3 Genomic DNA extraction from human blood

DNA extractions were performed by using the reagents in the BACC3 Nucleon DNA extraction kit (Amersham Biosciences).

Blood samples (4-5ml) of the patients involved in the phase I study (see previous paragraph) were thawed at room temperature before extraction, which was performed accordingly to the manufacturer's instructions. At the end of the extraction, DNA was precipitated by adding 2 volumes of cold absolute ethanol and then washed by adding cold 70% (v/v) ethanol. DNA was air dried and re-dissolved in appropriate volume of water.

DNA was quantified with Nano-Drop ND-1000 Spectrophotometer, according to the user's manual.

3.3 METHYLATION ANALYSIS

3.3.1 Sodium bisulfite modification of DNA

The methylation status of a DNA sequence can be determined using sodium bisulfite. Incubation of DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged (fig. 3.3.1, A). Therefore, bisulfite treatment gives rise to different sequences for methylated and unmethylated DNA (fig.3.3.1, B). The most critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. Cytosine forms adducts across the 5-6 bond with bisulfite; hydrolytic deamination of the resulting cytosine-bisulfite derivative gives a uracil-bisulfite derivative, and removal of the sulphonate group by a subsequent alkali treatment gives uracil. This reaction does not occur if the cytosine is methylated.

It is important to note that bisulfite reacts with cytosine either as the free base, the nucleoside (ribo- or deoxyribo-), the nucleotide or in oligonucleotides. However the reaction is highly single-strand specific (Clark et al., 1994).

Genomic DNA (1µg) was modified using EpiTect® Bisulfite Kit (Qiagen), according to the manufacturer's instructions. After modification, the DNA was eluted into 40µl Tris-EDTA, purchased with the kit.

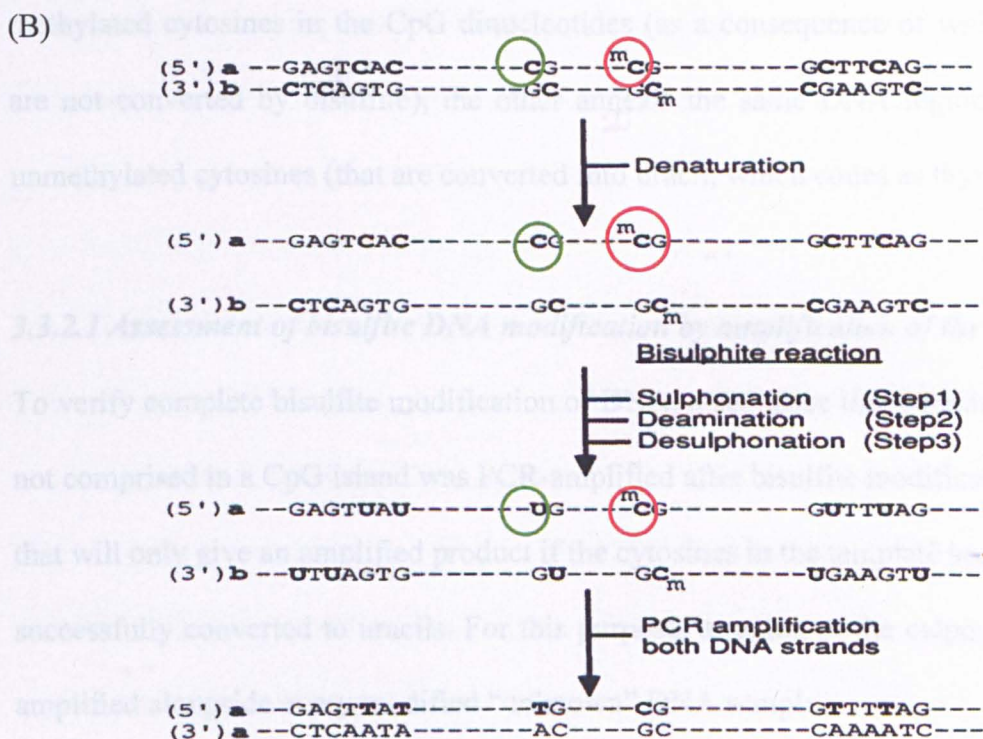
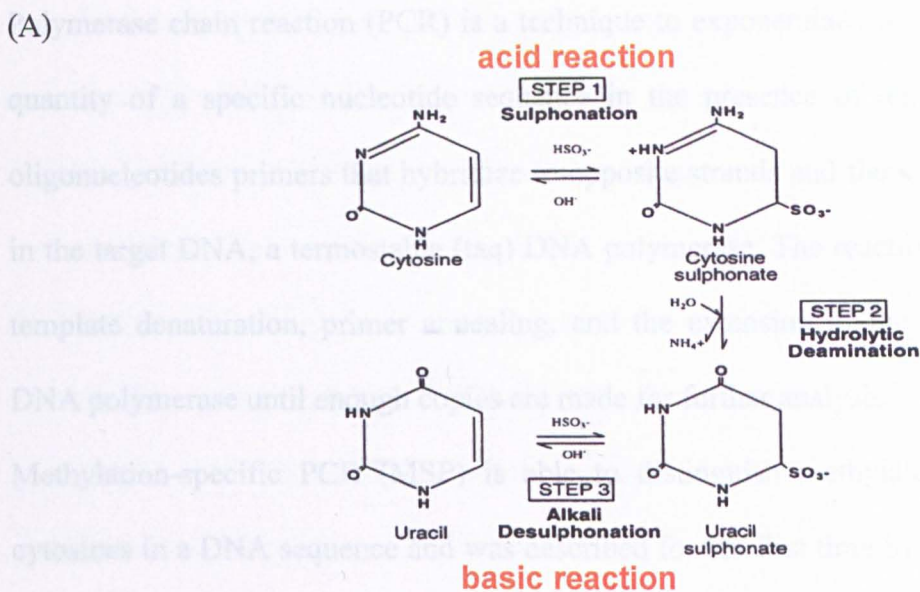


Figure 3.3.1 Sodium bisulfite reaction with cytosine residues

(A) bisulfite modification of an unmethylated cytosine

(B) Bisulfite modification in a DNA duplex: the green ring highlights one unmethylated and bisulfite converted cytosine and the red ring highlights one methylated and unconverted cytosine.

From (Clark et al., 1994), modified.

3.3.2 Amplifications of bisulfite modified genomic DNA by Methylation Specific PCR

Polymerase chain reaction (PCR) is a technique to exponentially amplify in vitro a small quantity of a specific nucleotide sequence in the presence of template sequence, two oligonucleotides primers that hybridize to opposite strands and flank the region of interest in the target DNA, a thermostable (taq) DNA polymerase. The reaction is cycled involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase until enough copies are made for further analysis.

Methylation-specific PCR (MSP) is able to distinguish methylated and unmethylated cytosines in a DNA sequence and was described for the first time by Hermann (Herman et al., 1996). It is based on the amplification of a selected region of DNA, previously modified by bisulfite, using two pairs of primers: one anneals a DNA region if this contains methylated cytosines in the CpG dinucleotides (as a consequence of which the cytosines are not converted by bisulfite), the other anneals the same DNA region if this contains unmethylated cytosines (that are converted into uracil, which codes as thymine).

3.3.2.1 Assessment of bisulfite DNA modification by amplification of the calponin gene

To verify complete bisulfite modification of DNA, a sequence that contains only cytosines not comprised in a CpG island was PCR-amplified after bisulfite modification with primers that will only give an amplified product if the cytosines in the template sequence have been successfully converted to uracils. For this purpose, a region of the calponin promoter was amplified alongside every modified “unknown” DNA sample.

PCR was done in a total volume of 25µl, containing 1µl (100ng) modified template DNA, 10µM of each primer (F: 5'-GGAAGGTAGTTGAGGTTGTG; R: 5'-CCCAAACCTCAAACTCTAACCTAAC), 3mM MgCl₂, 0.2mM deoxynucleotide triphosphates (Applied Biosystems, Warrington, United Kingdom), and 1 unit FastStart Taq (Roche Diagnostics, Lewes, United Kingdom). PCR amplification involved an initial incubation at 95°C for 5 minutes, followed by 35 cycles of: 95°C for 30 seconds, annealing

at 63°C for 30 seconds and extension at 72°C for 90 seconds. The annealing temperature was about 3-5 degrees lower than the melting temperature (T_m) of the primers calculated as follow: $T_m = 4X (G+C) + 2X (A+T)$. Following these 35 cycles of amplification, a final extension was done at 72°C for 10 minutes. After adding the loading buffer (EDTA 0.1M, sucrose 50%w/v, bromophenol blue solution 0.25% and xylene cyanol solution 0.25% to a final volume of 5ml with pure water) to each sample, PCR products were loaded on 2%w/v agarose (Eppendorf, Italy) gel in TAE buffer (Tris base 2M, EDTA pH 8 50mM, 5.1% glacial acid acetic to 1l with deionized water), together with 5µl of 50bp DNA ladder (ready-to-use marker 0.1mg/ml, Fermentas-MBI, Italy). PCR products (333bp) were visualized under UV light after ethidium bromide (5µg/ml solution in TAE, protected from light and stored at 4°C, Sigma. **Warning:** ethidium bromide is a mutagenic substance and should be handled with protective clothing and disposal gloves) staining.

Samples successfully modified by bisulfite give well defined amplification products.

3.3.2.2 Methylation Specific PCR of GSTP1 promoter

Primers for the GSTP1 gene promoter were designed described in Results (fig.4.2.6). PCR was done in a total volume of 25µl, containing 1µl (100ng) modified template DNA, 10µM of each primer (F: 5'-AGTTGCGCGGCGATTTC; R: 5'-GCCCAATACTAAATCACGACG), 2mM $MgCl_2$, 0.2mM deoxynucleotide triphosphates (Applied Biosystems, Warrington, United Kingdom), and 1 unit FastStart Taq (Roche Diagnostics, Lewes, United Kingdom).

PCR reaction was subjected to initial incubation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 30 seconds. The annealing temperature was determined as described in the previous paragraph. Final extension was done by incubation at 72°C for 10 minutes. The

amplification products were 140bp in length and were separated according to the previous conditions.

Because MSP is a sensitive assay for the analysis of DNA methylation, care has to be taken to minimize false-positive and false-negative PCR products. For this purpose, commercially available DNAs were used as template for setting the PCR conditions with the GSTP1 primers: In Vitro Methylated (IVM, CpGenome™ Universal Methylated DNA, Chemicon) is an enzymatically methylated human male genomic DNA and was used as a methylation-positive control (100% methylation); human male genomic DNA (Promega) was used as negative control (0% methylation).

3.3.2.3 Methylation Specific PCR of GSTM1 promoter

Primers for the GSTM1 gene promoter were designed as described in Results (fig.4.3.5); the region was amplified by using the touch down PCR, which allows the use of very high annealing temperatures (close to the melting temperature of the primers, calculated as described in the paragraph 3.3.2.1) in order to avoid false positives. For GSTM1 promoter amplification 2 couples of primers were used, specifically annealing methylated or not methylated bisulfite treated DNA (see figure 4.3.5) (Lodygin et al., 2005). The PCR conditions are summarized in the following table:

Table 3.3.1 MSP conditions for GSTM1

| | Primer Sequence | Product | Annealing | [Mg ⁺⁺] |
|-------|--|----------|--|---------------------|
| | Forward (F)/Reverse (R) 5'→3' on bisulfite modified template sequence | Size(bp) | Temp (°C) | mM |
| GSTM1 | F (M): GAAGTTGGCGAGGTCGAGTTTC | 157(M) | <u>Touch Down</u> 95°C x5min 95°C x 30'' 68°C x 30'' 2cycles 72°C x 60'' | 3 |
| | F (U): GGGAAGTTGGTGAGGTTGAGTTTT | | | |
| | R (M): ACCCGCCACAACCCGAAAAACG | 161(U) | 95°C x 30'' 66°C x 30'' 2cycles 72°C x 60'' | |
| | R (U): CAACCCACCACAACCCAAAAAACA | | 95°C x 30'' 65°C x 30'' 36cycles 72°C x 60'' 72°C x 10' | |

PCR products were separated and visualized accordingly to the conditions described in the previous paragraphs.

3.3.3 Pyrosequencing: general description

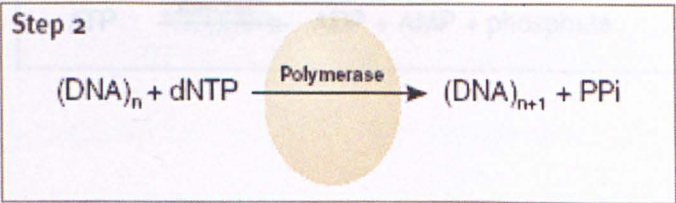
Pyrosequencing (PSQ) is a method that quantitatively monitors the incorporation of nucleotides through the enzymatic conversion of released pyrophosphate to generate a proportional light signal. Analysis of DNA methylation patterns by PSQ measures the degree of methylation at all CpGs within the sequenced region, after bisulfite treatment of DNA (Tost and Gut, 2007; Agah et al., 2004; Ahmadian et al., 2006).

Step 1

A sequencing primer is hybridized to a single stranded, PCR amplified, bisulfite treated DNA template, and incubate with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5’phosphosulfate (APS) and luciferin.

Step 2

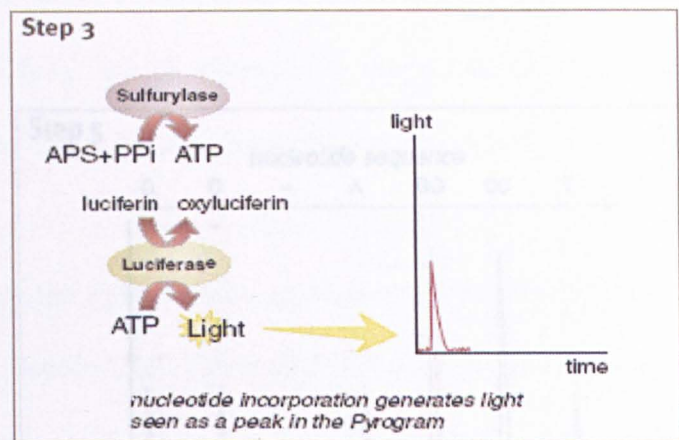
The first of four deoxyribonucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribonucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.



Step 3

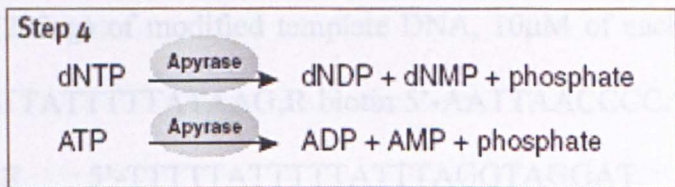
ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5’ phosphosulfate (APS). This ATP drives the luciferase mediated conversion of luciferin to

oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase –catalyzed reaction is detected by a charge



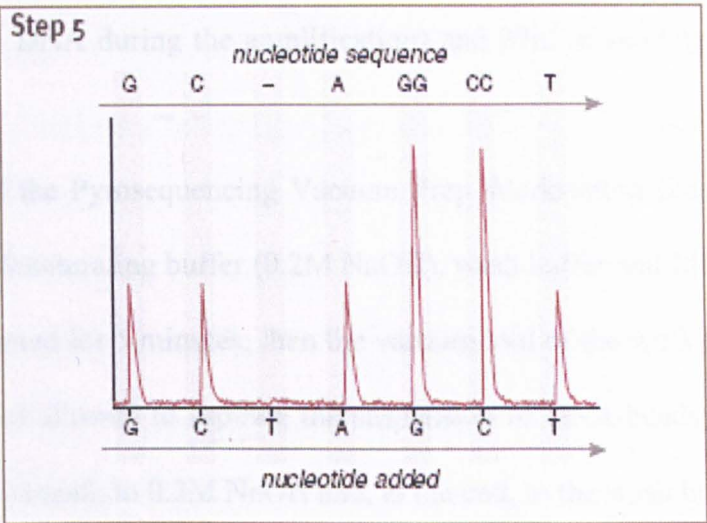
coupled device camera (CDD) and is seen as peak in a Pyrogram TM. The height of each peach (light signal) is proportional to the number of nucleotides incorporated.

Step 4 Apyrase, a nucleotide degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This switches off the light and regenerates the reaction solution. The next dNTP is then added.



Step 5 The addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP α S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not

recognized by luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram.



3.3.3 Pyrosequencing: *GSTP1* and *MAGE1A* promoter methylation analysis

To generate a sufficient amount of template, DNA was PCR-amplified, with one of the two primers labelled with biotin at its 5'-terminus. The primers chosen for this reaction amplifies all states irrespective of methylation status, because they anneal regions mainly devoid of CpG dinucleotides (fig.4.2.6). PCR was done in a total volume of 50µl, containing 2µl (200ng) of modified template DNA, 10µM of each primer (*GSTP1*:F 5'-GGGGAGGGATTATTTTATAAG,R biotin 5'-AATTAACCCCATACTAAAACTCT; *MAGE1A*: F 5'-TTTTTATTTTATTAGGTAGGAT, R biotin 5'-TCTAAAAACAACCCAACTAAAAC. All primers were purchased by VH Bio Ltd, UK), MgCl₂ 2mM for *GSTP1* and 4mM for *MAGE1A*, 0.2mM deoxynucleotide triphosphate (Applied Biosystems, Warrington, United Kingdom), and 2 units FastStart Taq (Roche Diagnostics, Lewes, United Kingdom).

PCR amplification was subjected to initial incubation at 95°C for 5 minutes, followed by 49 cycles of 95°C for 30 seconds, annealing at 54°C (*GSTP1*) or 53°C (*MAGE1A*) for 30

seconds and 72°C for 30 seconds. Final extension was done by incubation at 72°C for 10 minutes.

In a 96 well PCR plate, PCR products were dispensed. In each well, containing the amplified DNA (40µl), 3µl of streptavidin coated beads (which bind the biotinylated primer inserted into DNA during the amplification) and 37µl of binding buffer (Biotage) were added.

The four troughs of the Pyrosequencing Vacuum Prep Workstation (Biotage) were filled with 70% Ethanol, denaturing buffer (0.2M NaOH), wash buffer and Milli-Q water.

PCR plate was vortexed for 5 minutes; then the vacuum tool of the workstation was placed in the PCR plate and allowed to aspirate the suspension of DNA-beads; the vacuum tool was moved to 70% ethanol, to 0.2M NaOH and, at the end, to the wash buffer.

The described steps allow to prepare the single-stranded DNA template (through the alkali treatment) immobilized on streptavidin-coated sepharose beads.

In a PSQ 96 Low Plate the sequencing mix, consisting of 1.5µl sequencing primer 10µM (GSTP1: GGATTATTTTATAAGGT; MAGE1A: TGTTGTTAGTTTTGGTTTAT) and 43.5µl annealing buffer (Biotage) for each sample, was dispensed. The sequencing primer in the PSQ plate was then hybridized to the single-stranded DNA template.

After a 5 minute incubation at 80°C, the PSQ 96 plate was transferred to the PSQ 96MA pyrosequencer (Biotage).

All the reagents (nucleotides, substrate and enzyme mix) for the pyrosequencing were dispensed into the appropriate wells of the PSQ TM⁹⁶ Reagent Cartridge, according to the volume calculation of the software. The pyrosequencer automatically dispensed these reagents to the samples in the PSQ plate, when the reaction was allowed to run.

3.4 IN VIVO EXPERIMENTS

3.4.1 Animals

CB17/SCID (Severe Combined Immunodeficiency) male mice (Charles River, Calco, Lecco, Italy) were used for LNCaP cell transplantations. Eight week old mice, weighing 20-25g, were maintained under specific pathogen-free conditions, in cages with paper filter covers, and they were provided food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116, G.U., suppl.40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec 12, 1987; Guide for the use of Laboratory Animals, United States National Research Council, 1996).

3.4.2 Tumour model

For drug testing, LNCaP cells were suspended in saline and BD Matrigel Matrix Basement membrane (BD Biosciences) (ratio 1:1) and implanted subcutaneously into the left flank of recipient SCID mice (5×10^6 cells/mouse). When the tumour was palpable (approximately 0.2g weight, attained roughly three weeks after transplant), animals were randomly divided into test groups each consisting at least of seven mice.

3.4.3 Drug administration

Brostallicin was administrated intravenously (iv) and zebularine was administrated intraperitoneally (ip) according to the schedule indicated in Results (fig.4.2.2). The volume of administration for both drugs was 10ml/kg of body weight.

Zebularine was dissolved in saline and 50mg/ml stock solutions were prepared and stored at -20°C, while brostallicin was prepared immediately before use: it was dissolved in

DMSO 50% in saline at 1mg/ml and then diluted in saline to the final concentration 0.04mg/ml.

3.4.4 Evaluation of antitumour activity and toxicity

Beginning on day 0, the length (L) and width (W) of the solid tumour mass were measured using callipers twice weekly and the tumour volume (TV) was calculated as $TV = (L \times W^2)/2$ (assuming a density of 1, the calculated tumour volume corresponds to the tumour mass value). The tumour volume at day n was expressed as relative tumour volume (RTV) according to the formula $RTV = TV_n/TV_0$, where TV_n is the tumour volume at day n and TV_0 is the tumour volume at day 0.

The doubling time (days) of the not treated tumours was calculated as $DT = \ln 2/\lambda$, where λ is the coefficient of the X value in the equation of the exponential curve that was fitted the tumour growth curve of each control mouse.

The percentage of tumour growth inhibition (T/C%) was determined by calculating RTV as: $T/C\% = 100 \times (\text{mean RTV of treated group})/(\text{mean RTV of control group})$. According to the National Cancer Institute standards, a $T/C \leq 42\%$ is the minimum level for a drug to be considered active (Geran RI et al.1972).

Toxicity was evaluated on the basis of weight loss. Body weight was recorded the same day of tumour measurement and was taken as a measure of toxicity, together with gross autopsy findings. The percentage of weight loss was calculated by the ratio:

$$100 \times (\text{mean Body Weight Treated Group} / \text{mean Body Weight Control Group}).$$

Antitumour activity was evaluated at the highest non-toxic dosage of the drugs administrated as single agents, which is the highest dosage that can be administrated without causing death or undue toxicity. A dose producing a weight loss nadir of 20% or drug related death was considered as excessively toxic.

3.4.5 Statistical analysis

Tumour volumes of treated and untreated mice were compared by analysis of variance (ANOVA) using the Statview Statistical Package (SAS Institute Inc., third edition).

3.4.6 GSTpi expression

For evaluation of GSTpi expression, a group of 5 mice was treated with the same scheduled used for antitumour activity, that is with two cycles (each of 8 daily treatments) of zebularine 500mg/kg.

The day after the last treatment with zebularine, mice were sacrificed, tumour fragments were excised and immediately frozen in dry ice and stored at -80°C for western blotting or fixed 24h in formalin (4% in PBS, Bio-Optica) for immunohistochemistry.

3.4.6.1 Immunohistochemistry

Immunohistochemistry on LNCaP tumors was kindly performed for me by Dr. Roberta Ceruti at Nerviano Medical Sciences (Nerviano, Milan, Italy). Formalin fixed tumours were embedded in paraffin. Slices (4µm) were put on super frost plus slides. Haematoxylin and eosin staining was performed for morphological evaluation. For immunohistochemistry, a Ventana Discovery autostainer was used. Briefly, standard CC1 (sCC1) unmasking protocol was used, followed by GSTpi monoclonal antibody (Novocastra NCL-GST pi-438, dilution 1:50 in D-PBS) incubation for 2h. The En-Vision labelled Polymer-HRP anti mouse (DAKO K4001) was used as secondary antibody: incubation was for 16 minutes. Standard diaminobenzidine (DAB) chromogen from Ventana revealed the antibody binding.

3.5 WESTERN BLOTTING

Western blotting analysis involves the separation of proteins according to molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), electrophoretically transferring them to a membrane and then using specific antibodies to detect the protein of interest.

3.5.1 Protein extract preparation

Total proteins were extracted from cells growing in culture by a lysis method. Basically cell cultures were washed twice with ice-cold PBS and then detached with a disposable scraper to 500 µl of PBS. The suspension was then centrifuged at 1,200rpm for 10 minutes at room temperature and after centrifugation, the pellet was resuspended in an amount of lysis buffer (Tris-Cl pH8 50mM, NaCl 150mM, EGTA 1mM, NaF 100mM, glycerol 10%, MgCl₂ 1mM, Triton X-100 1% + protease inhibitor cocktail (Sigma) containing a mixture of protease inhibitors, in particular aprotinin, bestatin hydrochloride, EDTA, leupeptin, specifically inhibiting serine proteases, aminopeptidases, metalloproteases and cysteine proteases) dependent on the size of cell pellet (about 100µl for 5x10⁶ pelleted cells) and incubated on ice for 30 minutes in an orbital shaker. Cellular debris was pelleted by centrifugation at 12,000rpm at 4°C for 10min, and the supernatant was recovered.

Frozen tissue fragments were homogenized using a Polytron homogenizer in lysis buffer (ratio 1:1 w/v). Insoluble material was pelleted at 12000rpm for 30 minutes at 4°C and the supernatant was recovered and placed in a fresh Eppendorf tube (1.5 ml).

An aliquot (2 µl) of total proteins extracted from both cells and tumour fragments was used for determination of protein concentration.

3.5.2 Determination of protein concentration

Before determining the protein concentration of the samples examined, a calibration curve was prepared. Solutions of bovine serum albumin (BSA) (ranging between 1 and 20 μ g/200 μ l) were prepared from a stock solution of BSA obtained by dissolving powdered BSA (Sigma) in water. In a 1.5ml tube, 200 μ l of each BSA solution were mixed with 600 μ l of distilled water and 200 μ l of BioRad protein assay dye (BioRad). In the blank sample 800 μ l of distilled water was mixed with 200 μ l of BioRad protein assay dye. Samples were rapidly transferred into disposable cuvettes (PBI International, Milan-Italy) and the absorbance at 595nm was measured in the spectrophotometer. The absorbance value corresponding to the blank sample was subtracted from the values obtained in the BSA-containing samples. Each calibration sample was run in triplicate. The calibration curve obtained in such a way, allows extrapolation of the exact absorbance value of 1 μ g of proteins present in the solution.

Protein concentration in the cellular or fragment extract was determined according to the Bradford protocol. In a 1.5ml tube, 2 μ l of protein extract was mixed with 200 μ l of BioRad protein assay dye and distilled water to a final volume of 1ml. The absorbance at 595nm was measured in the spectrophotometer and the sample concentration was calculated from the calibration curve previously determined.

3.5.3 SDS-PAGE

An aliquot (typically 30 - 60 μ g) of protein of each sample was mixed with the same volume of SDS loading buffer (100mM Tris-HCl pH 8.8, 200mM DTT, 4%w/v SDS, 20%v/v glycerol and 0.2% bromophenol blue in sterile water).

The mixture was heated at 95°C for 5 min and allowed to cool. Samples were subjected to SDS-PAGE.

Stacking (125mM Tris-HCl pH 6.8, 0.1%w/v SDS, 5%w/v acrylamide/bis 37.5:1, 0.05% w/v ammonium persulphate and 5 μ l TEMED to a final volume of 10ml with deionized water) and resolving (750mM Tris-HCl pH 8.8, 0.1%w/v SDS, X %w/v acrylamide/bis 37.5:1, 0.05% w/v ammonium persulphate and 7.5 μ l TEMED to a final volume of 10 ml with deionized water) gels were prepared shortly before pouring. Ammonium persulphate catalyses polymerization and TEMED accelerates the reaction and so these two reagents were added last with thorough mixing before the addition.

Proteins were resolved on a minigel apparatus (BioRad) and run for 2h at 100V in TGE buffer (25mM Tris base, 192mM glycine, 0.1% w/v SDS to a final volume of 5l with deionized water. Stored at room temperature).

The progress of the electrophoresis was monitored using pre-stained molecular weight markers (11-170 KDa, Page Ruler Prestained Protein Ladder, Fermentas).

3.5.4 Protein transfer and detection

The separated proteins were transferred onto a PVDF (polyvinylidene fluoride) membrane (Millipore), at 400mAmpere (corresponding to about 120V) for 45 minutes on ice, using a BioRad Mini transfer blot in transfer buffer (50mM Tris base, 100mM glycine, 0.01% w/v SDS, 20% v/v methanol to a final volume of 4l with deionized water. The solution was stored at room temperature).

Filters were stained with Ponceau red solution (0.1% w/v PonceauS in 5% v/v acetic acid, Sigma) to check sample loading and transfer and then washed in methanol. Blots were exposed for 2h at room temperature to the desired primary antibodies diluted to the optimal working solution in 5% non-fat dried milk dissolved in TBS-T 0.1% (Tris base 20mM, NaCl 137mM, 0.1% v/v Tween to a final volume of 1l with deionized water. The pH was adjusted to 7.6 with concentrated HCl and stored at 4°C).

All the following procedures were carried out at room temperature on a shaker. After incubation, the blots were washed twice with TBS-T 0.1% for 10-30 minutes and

incubated with the appropriate horseradish-peroxidase linked anti-mouse or anti-goat IgG secondary antibody (SantaCruz Biotechnology) for 1h using appropriate dilutions. Blots were washed again and detection was performed with an enhanced chemiluminescent detection system (ECL, GE Healthcare). Horseradish peroxidase acts as a catalyst for the oxidation of a luminol substrate, which subsequently emits small but sustained quantities of light. This chemiluminescence is specifically enhanced by chemical enhancers such as phenols. This has the effect of increasing the light output approximately 1000 fold and extending the time of light emission, allowing an image to be recorded on photographic film. The blots were exposed to film for different times ranging from 15 seconds to 3 minutes and developed using an X-o graph compact x-2 developer with Kodak GBX developer and fixer.

The antibodies used for Western blot analysis in this study were NCL-GSTpi 438 mouse monoclonal antibody for GSTpi (supplied by Novocastra) and, as actin was used as housekeeping gene, actin goat polyclonal antibody (SantaCruz Biotechnology).

Stock solutions were stored at 4°C and working solutions were prepared by diluting the antibody 1:500 in 5% non-fat dried milk dissolved in TBS-T 0.1%.

Horseradish peroxidase-coupled secondary antibodies (goat anti-mouse for GSTpi and donkey anti-goat for actin IgGs) were supplied by Santa Cruz and diluted 1:3000 in 5% non-fat dried milk dissolved in TBS-T 0.1%.

3.6 Assay principle of total GST Enzymatic Activity

In vitro enzymatic activity of total GST was measured according to the method of Habig and Jackoby (Habig and Jakoby, 1981). The reaction was initiated by the addition of 1-chloro-2,4-dinitrobenzene (CDNB) and the velocity was determined by measuring the change in absorbance at 340 nm in 1 minute due to the 2,4-dinitrophenylglutathione formation and subtracting the rate of non-enzymatically-mediated conjugation (i.e. in the absence of enzyme). One unit of enzymatic activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of S-2,4-dinitrophenylglutathione per min at 23°C. Data are expressed as nanomoles of dinitrophenylglutathione formed /min/mg of total protein using the extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

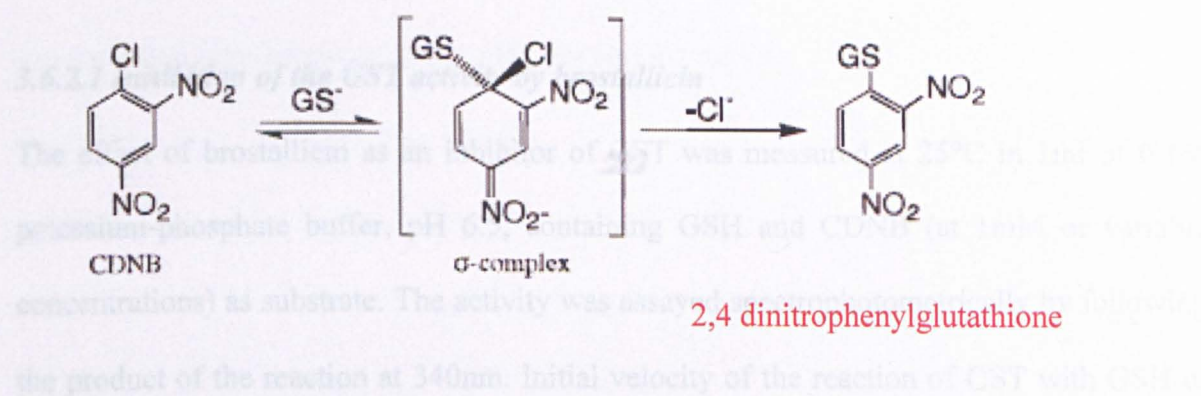


Figure 3.6.1 Reaction of GSH with CDNB catalyzed by GST family of enzymes

The addition of GSH to CDNB is a nucleophilic aromatic substitution reaction that occurs via an addition-elimination sequence involving a short-lived σ -complex intermediate and leading to 2,4 dinitrophenylglutathione formation (Armstrong, 1997).

3.6.1 Total GST activity on cell LNCaP and Du145 cells

Cells were washed twice with ice-cold PBS, harvested, lysed in sterile ice-cold water by sonication to destroy cellular membranes and centrifuged at 15000rpm, 4°C for 10 minutes. The supernatant was transferred into a 1.5 ml Eppendorf tube, and an aliquot was used for protein quantitation, by the BioRad protocol. An aliquot (100 μl) of supernatant

was incubated with 100 μ l of phosphate buffer (K_2HPO_4 1M, KH_2PO_4 1M to pH 6.5) and 1 μ mole of glutathione (GSH, 100 μ l of a solution of 10 mM GSH in water) in a final volume of 1ml with sterile water for 3 min at 25°C. At the end of the incubation, 1 μ mole of CDNB (20 μ l of 50 mM CDNB solution in ethanol. CDNB is purchased as dry powder from Sigma) was added, the mixture was transferred to a 1ml microcuvette and the kinetics of CDNB conversion followed by spectrophotometer (UVIKON-860, Kontron Instruments). Data were normalised for the amount of protein present in each sample.

3.6.2 KINETIC ASSAYS

3.6.2.1 Inhibition of the GST activity by brostallicin

The effect of brostallicin as an inhibitor of GST was measured at 25°C in 1ml of 0.1M potassium-phosphate buffer, pH 6.5, containing GSH and CDNB (at 1mM or variable concentrations) as substrate. The activity was assayed spectrophotometrically by following the product of the reaction at 340nm. Initial velocity of the reaction of GST with GSH or CDNB was measured in the presence of different concentrations of brostallicin (0 – 50 μ M for GSTP1 and 0 - 1 μ M for GSTM). At each inhibitor concentration, either CDNB or GSH was varied from 0.25 to 2mM, while the co-substrate was maintained constant at 1mM. Initial velocity data were reported as double reciprocal plots.

Apparent inhibition constant (K_i) were calculated by re-plot analysis of the primary reciprocal plots. With GST μ , the re-plot analysis, obtained when GSH was the varied substrate, gave a non-linear trend. In this case, the inhibition constant was obtained by a plot of $1/\Delta$ slope and $1/\Delta$ intercept versus $1/\text{inhibitor concentration}$. Δ slope and Δ intercept are the slope and the intercept obtained at a given brostallicin concentration subtracted of the slope or the intercept obtained in the absence of brostallicin.

(Segel, I.H., (1975) New York: Wiley – Interscience).

3.6.2.2 *GST activity with brostallicin*

GST activity, with GSH and brostallicin as substrates, was measured in 1ml of 0.1M potassium phosphate buffer, pH 6.5, using a spectrofluorometer (Perkin Elmer) with a sample holder set at 25°C. Excitation wavelength was 370nm and emission wavelength was at 425nm.

The dependence of initial velocity on substrate concentration was studied using 1μM GSTP1 or GSTM and varying brostallicin concentration from 1μM to 100μM. The increase of fluorescence intensity was recorded in a time range of one minute (ΔF/min).

Kinetic parameters were determined by fitting the Hill equation:

$$V = V_{\max} \cdot S^{n_H} / (1 + (K^{n_H} / S^{n_H}))$$

to the experimental data, where n_H is the Hill coefficient and K is the substrate concentration corresponding to the enzyme- half saturation.

4. RESULTS

CHAPTER 4.1

ANTITUMOUR ACTIVITY OF BROSTALLICIN ON HUMAN PROSTATIC CANCER CELLS: *IN VITRO* COMBINATION WITH DEMETHYLATING AGENTS

4.1.1 INTRODUCTION

The reversibility of the epigenetic modifications of DNA represents an opportunity in the therapeutic strategies against cancer: it offers the possibility to re-express molecular targets which exert a fundamental role in the antitumour activity of specific drugs. However, because methylation is being increasingly recognized as a means of acquisition of drug resistance, the combinations of cytotoxic molecules with agents reverting epigenetically silenced genes involved in the resistance mechanism should be considered as a novel treatment strategy.

The choice of the “right” demethylating agents to use in drug combinations represents a very important step in *in vitro* studies, i.e. testing their capacity to induce the re-expression of the target genes prior defining the most effective schedule for combination with the cytotoxic drug considered.

Prostate cancer has been widely investigated for epigenetic modifications of its genome, especially for the strong methylation affecting the GSTP1 promoter (Li et al., 2004; Millar et al., 1999), starting from the first stages of tumour initiation. For this reason prostate cancer cell lines (which have been shown to retain the methylation of specific genes) have been extensively used in order to determine the capacity of nucleoside or non nucleoside inhibitors of methylation to re-express methylated genes. Among these, GSTP1 has been one of the most frequently analyzed. GSTP1 methylation presented the first suggestion of the possibility of combining demethylating drugs with the minor groove binder, brostallicin, a pro-drug that needs to be bound to GSH before exerting its cytotoxicity.

Here, I will describe the different combinations of demethylating molecules and brostallicin on the LNCaP prostate cancer cell line, which retains the methylation of the GSTP1 promoter found in primary prostatic tumours (Song et al., 2002).

4.1.2 RESULTS

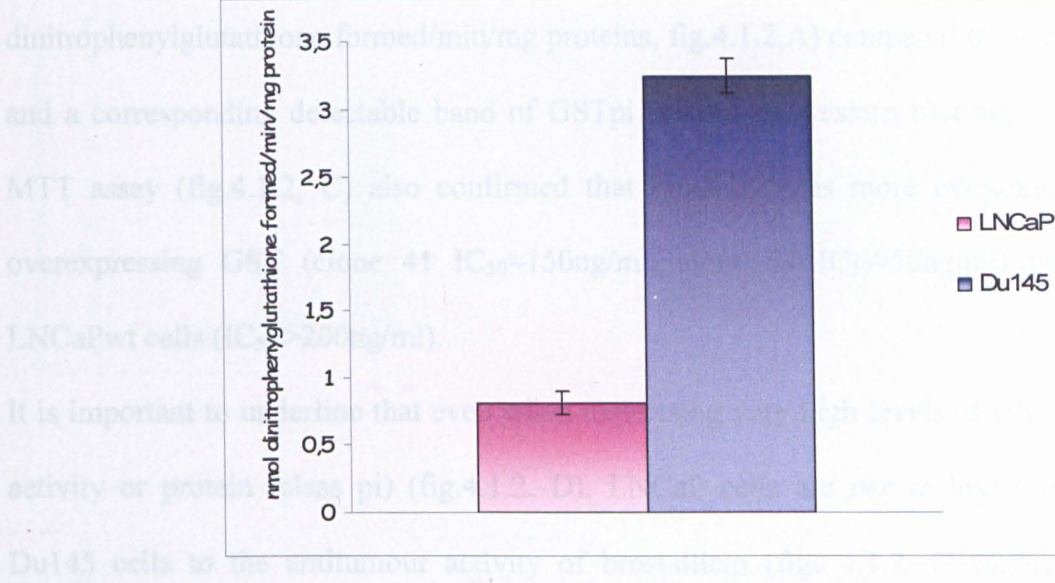
4.1.2.1 Differential toxicity of brostallicin on LNCaP and DU145 prostatic cancer cell lines

To evaluate the relationship between GST expression and antitumour activity of brostallicin on prostatic tumour models I used the two prostatic cancer cell lines, Du145 and LNCaP. LNCaP do not express GSTpi, Du145 cells do; thus, when the total GST activity was assessed using CDNB as substrate, 0.8nmol dinitrophenylglutathione was formed/min/mg protein in cytosolic extracts obtained from LNCaP cells and 3.2nmol dinitrophenylglutathione was formed/min/mg proteins in cytosolic extracts obtained from Du145 cells (fig. 4.1.1, A) respectively. This was confirmed by western blotting, using antibodies specific for GSTpi, which shows an easily detectable level of GSTpi expression in Du145 and no expression at all in LNCaP cells (fig. 4.1.5, first and second lanes).

When the cytotoxicity of brostallicin was determined by using the MTT assay (fig.4.1.1, B), brostallicin was more active on Du145 cells ($IC_{50} \approx 30\text{ng/ml}$) than on LNCaP wild type cells ($IC_{50} > 150\text{ng/ml}$), supporting the contribution of GST to brostallicin activity.

In order to overcome the differences in the genetic background of these two cell lines, which prevent the evaluation of the specific contribution of GST alone to the antitumour activity of brostallicin, and to verify whether LNCaP cells can be sensitized to brostallicin by modulating only GST expression, LNCaP cells were transfected with a human GSTpi cDNA, subcloned in the pcDNA3 vector. The particular choice of the pi class was based on previous data obtained both *in vitro* and *in vivo* on the ovarian cancer cell line A2780, in which a strong correlation between the GSTpi levels and the antitumour activity of brostallicin was found (Geroni et al., 2002). However it should be noted that, in human tumours, GSTpi is the most abundant among the different classes of this family.

Following growth of selected clones in antibiotic (G418), LNCaP cells showed (A) substantial increase in total GST activity (expressed as



Further doses higher than the IC_{50} do not further increase the cytotoxicity of LNCaP-GST cells.

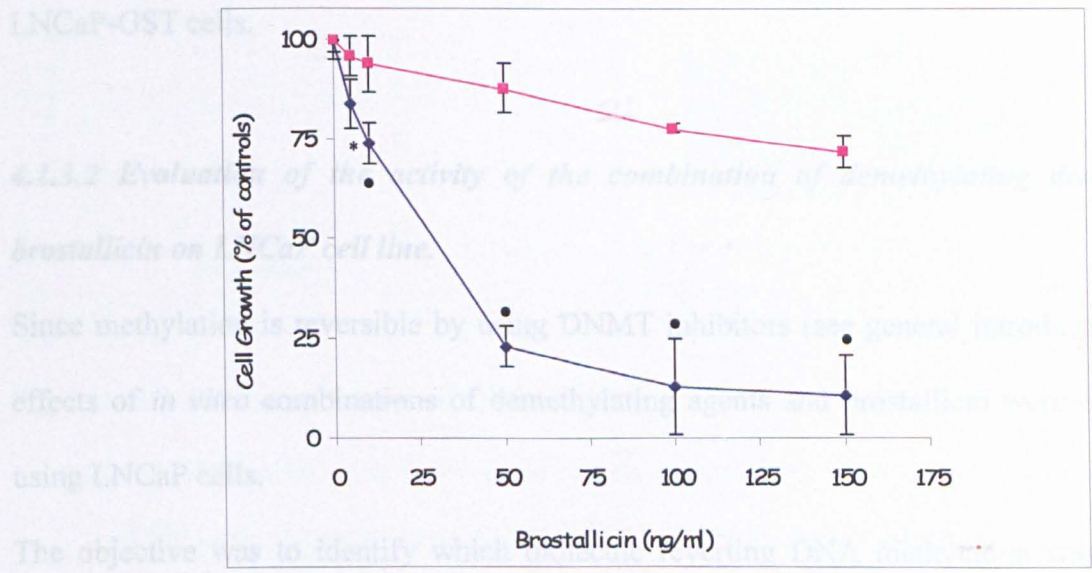


Figure 4.1.1 Analyses of LNCaP and Du145 cell lines

(A) total GST activity of cytosolic extracts of LNCaP and Du145 cells, as determined by CDNB assay. The boxes show the average values of two independent experiments, each consisting of three replicates, \pm SD (bars).

(B) effect of increasing concentrations of brostallicin on growth of LNCaP (pink line) and Du145 (blue line) cells as determined by MTT assay. The data shown are the mean of three independent experiments, each consisting of six replicates. Differences are statistically significant with $p < 0.05$ (*) at 5ng/ml brostallicin and $p < 0.001$ (●) from 10ng/ml to 150ng/ml brostallicin. Bars:SD.

Following growth of selected clones in antibiotic (G418), LNCaP-GSTpi clones 41 and 54 showed a substantial increase in total GST activity (respectively, ≈ 20 and ≈ 63 dinitrophenylglutathione formed/min/mg proteins, fig.4.1.2,A) compared to wild type cells and a corresponding detectable band of GSTpi protein by western blotting (fig.4.1.2,B). MTT assay (fig.4.1.2, C) also confirmed that brostallicin is more cytotoxic in clones overexpressing GST (clone 41 $IC_{50} \approx 150$ ng/ml, clone 54 $IC_{50} \approx 50$ ng/ml) than against LNCaPwt cells ($IC_{50} > 200$ ng/ml).

It is important to underline that even when expressing very high levels of GST enzymatic activity or protein (class pi) (fig.4.1.2, D), LNCaP cells are *per se* less sensitive than Du145 cells to the antitumour activity of brostallicin (figs 4.1.2, C versus 4.1.1, B). Furthermore doses higher than the IC_{50} do not further increase its cytotoxic activity in LNCaP-GST cells.

4.1.3.2 Evaluation of the activity of the combination of demethylating drugs with brostallicin on LNCaP cell line.

Since methylation is reversible by using DNMT inhibitors (see general introduction), the effects of *in vitro* combinations of demethylating agents and brostallicin were evaluated using LNCaP cells.

The objective was to identify which molecule reverting DNA methylation was able to increase the activity of brostallicin on LNCaP cells.

The most potent/effective demethylating drug known is the cytidine analog 5-aza-dC; as a consequence it was the first one I used in combination with brostallicin.

LNCaP cells were pretreated with $0.05 \mu\text{M}$ 5-aza-dC for 2 weeks (see Materials and Methods chapter, 3.1.4) and, after seeding in 6-well plates, treated with increasing concentrations of brostallicin for 72h. Initially I had used doses of 5-aza-dC currently adopted *in vitro*, in particular $10 \mu\text{M}$ for two weeks (Lin et al., 2001a), but this treatment

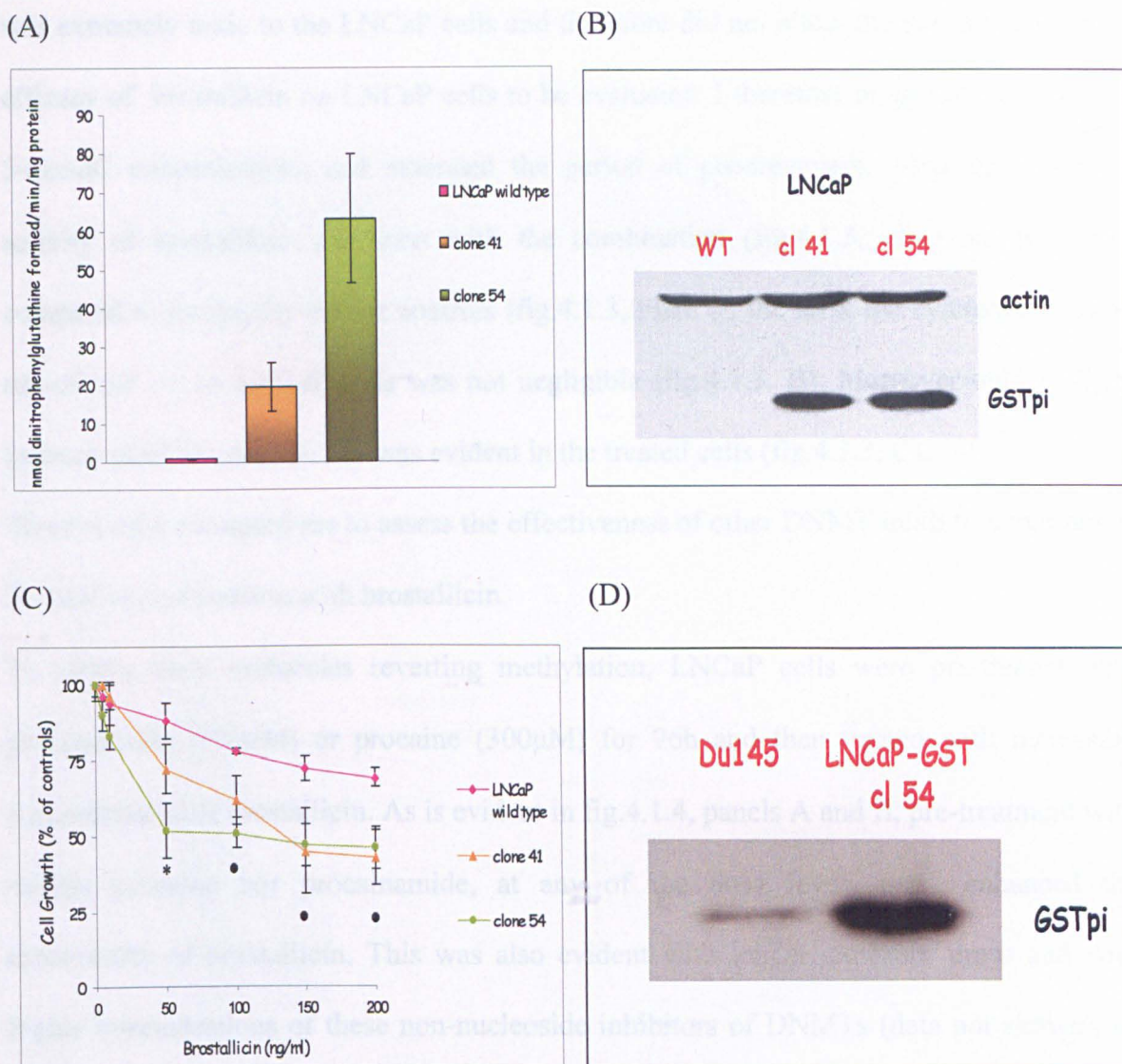


Figure 4.1.2 Characterization of LNCaP-GSTpi clones

(A) total GST activity of cytosolic extracts of the selected LNCaP-GST clones, as determined by CDNB assay. The boxes show the average of three independent experiments and the bars, S.D.

(B) western blotting analysis of two LNCaP-GSTpi transfected clones. 40µg of total proteins were loaded on a polyacrylamide gel and GSTpi detected using a monoclonal antibody. Anti-actin antibody was used to assess consistency of the gel loading and transfer.

(C) effect of increasing concentrations of brostallicin on LNCaPwt and LNCaP-GST clones as determined by MTT assay. The data are the mean of three independent experiments, each consisting of six replicates. Differences (for both clones versus controls) are statistically significant at brostallicin 50ng/ml (*, $p < 0.05$) and brostallicin ≥ 100 ng/ml (•, $p < 0.001$), as determined by the ANOVA test.

(D) western blotting analysis for GSTpi on 40µg of total proteins extracted by LNCaP-GST clone 54 and Du145 cells.

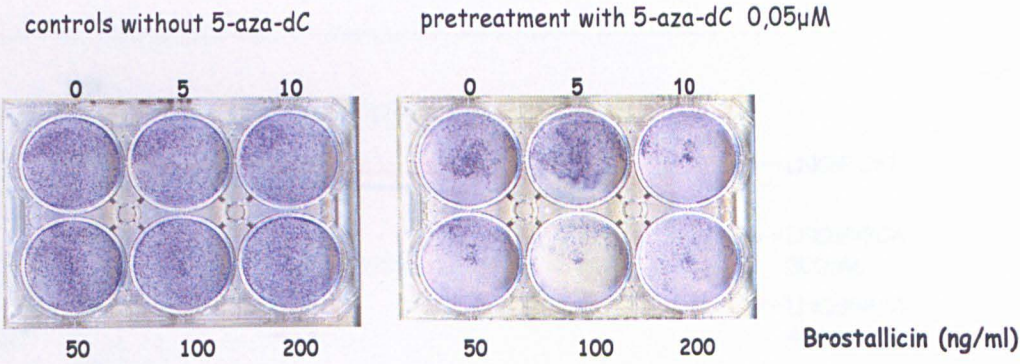
was extremely toxic to the LNCaP cells and therefore did not allow the possible increased efficacy of brostallicin on LNCaP cells to be evaluated. I therefore progressively lowered 5-aza-dC concentrations and extended the period of pre-treatment. Although a higher activity of brostallicin was seen with the combination (fig.4.1.3, plate on the right) compared to its activity on the controls (fig.4.1.3, plate on the left), the cytotoxicity of 5-aza-dC *per se* on LNCaP cells was not negligible (fig.4.1.3, B). Moreover only a slight increase of GST total activity was evident in the treated cells (fig.4.1.3, C).

These results prompted me to assess the effectiveness of other DNMT inhibitors that might be used in combination with brostallicin.

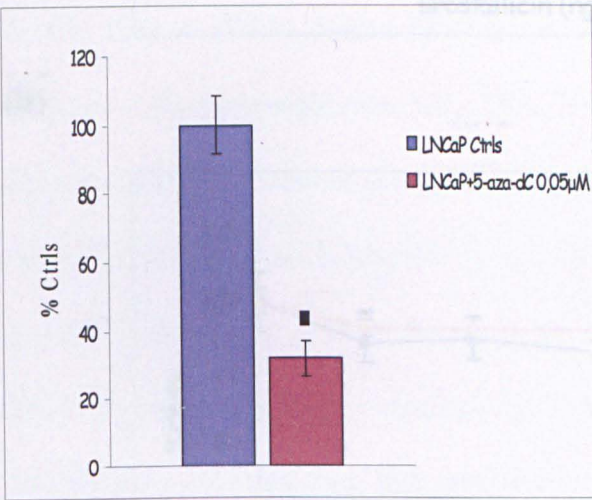
To assess other molecules reverting methylation, LNCaP cells were pre-treated with procainamide (100 μ M) or procaine (300 μ M) for 96h and then treated with increasing concentrations of brostallicin. As is evident in fig.4.1.4, panels A and B, pre-treatment with neither procaine nor procainamide, at any of the dose levels used, enhanced the cytotoxicity of brostallicin. This was also evident with longer exposure times and with higher concentrations of these non-nucleoside inhibitors of DNMTs (data not shown), so these combinations were not further considered.

The cytidine deaminase inhibitor zebularine is known to be less toxic and more stable than 5-aza-dC (Cheng et al., 2003) and this allows prolonged treatments without replacing the drug daily. These characteristics make zebularine particularly practical for combinations with cytotoxic drugs. When zebularine was used to pretreat LNCaP cells (96h of continuous treatment) before brostallicin, it clearly sensitized them to the cytotoxic drug and with increasing dose of zebularine there was a greater effect of the combination (fig.4.1.5, B). Although treatment with zebularine alone at 125 μ M showed detectable toxicity (fig.4.1.5, A), it produced the greatest enhancement of the cytotoxicity of brostallicin; the effects reached with 100 μ M and 125 μ M zebularine nearly reached those of brostallicin alone on LNCaP-GST clones. However, when I determined GSTpi expression,

(A)



(B)



(C)

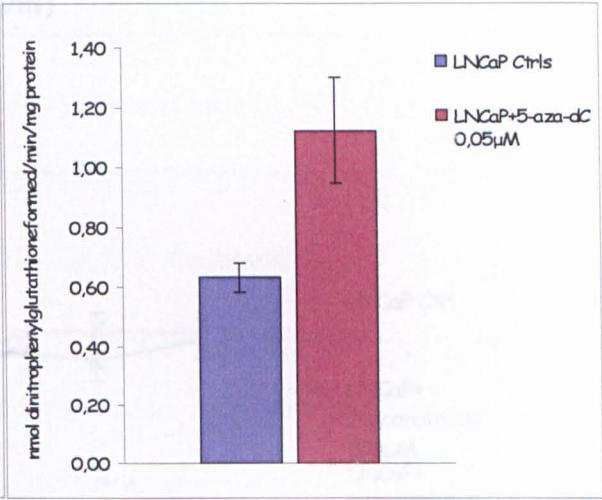


Figure 4.1.3 Effects of 5-aza-dC on LNCaP cells

- (A) Effect of a 2 week pre-treatment with 5-aza-dC (0.05µM) on brostallicin cytotoxicity in LNCaP cells. Cristal Violet staining was performed 72h after treatment with increasing concentrations of brostallicin as shown.
- (B) 5-aza-dC toxicity after 2 week treatment, as assessed by MTT assay. Cells were treated with 5-aza-dC in T-25 flasks before being seeded in 96 plates for the assay. The data shown are the mean of two independent experiments each consisting of six replicates. Difference between control and treated cells is statistically significant with $p < 0.01$ (■).
- (C) Total GST activity of cytosolic extracts of LNCaP treated for 2 weeks with 5-aza-dC, as determined by CDNB assay. The graphs were the average of two independent experiments. Bars, S.D.

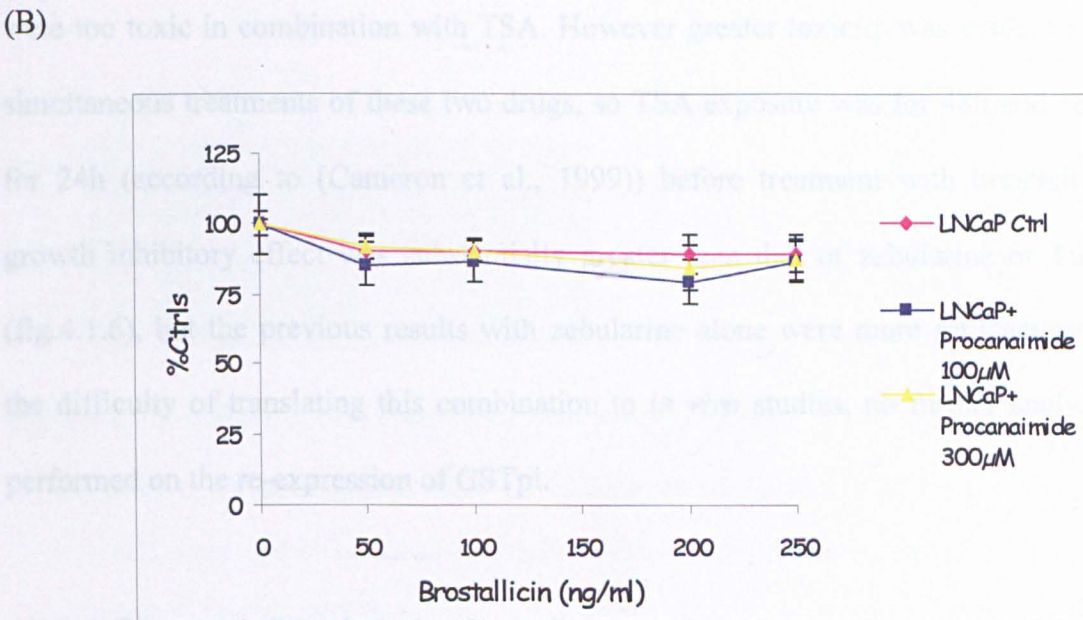
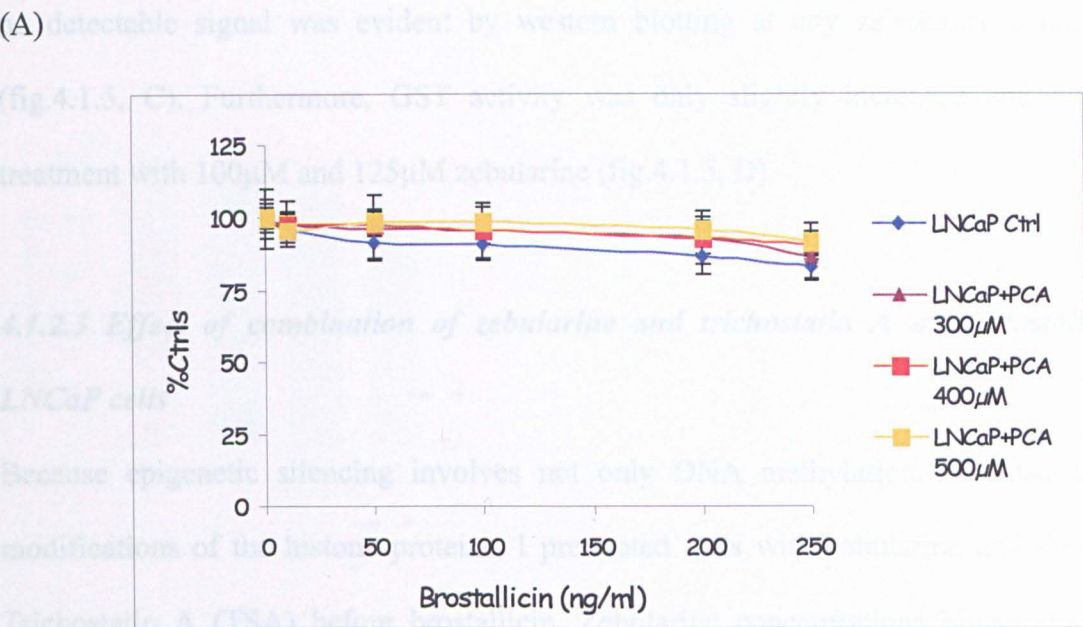


Figure 4.1.4 Effect of combination of procaine or procainamide and brostallicin on LNCaP cells

Effect of 72h treatment with increasing concentrations of brostallicin on LNCaP cells pretreated with procaine, (PCA, A) or procainamide (B) for 96h, as determined by MTT assay. Differences between control and treated groups are not statistically significant. Bars, S.D.

no detectable signal was evident by western blotting at any zebularine concentration (fig.4.1.5, C). Furthermore, GST activity was only slightly increased and only after treatment with 100 μ M and 125 μ M zebularine (fig.4.1.5, D).

4.1.2.3 Effect of combination of zebularine and trichostatin A with brostallicin on LNCaP cells

Because epigenetic silencing involves not only DNA methylation, but also chemical modifications of the histone proteins, I pretreated cells with zebularine and the HDACi Trichostatin A (TSA) before brostallicin. Zebularine concentrations higher than 50 μ M were too toxic in combination with TSA. However greater toxicity was evident following simultaneous treatments of these two drugs, so TSA exposure was for 48h and zebularine for 24h (according to (Cameron et al., 1999)) before treatment with brostallicin. The growth inhibitory effect was substantially greater than that of zebularine or TSA alone (fig.4.1.6), but the previous results with zebularine alone were more satisfactory. Due to the difficulty of translating this combination to *in vivo* studies, no further analyses were performed on the re-expression of GSTpi.

4.1.2.4 Effects of the zebularine-brostallicin combination on Du145 and LNCaP-GST cells

In the experiments I am going to describe, I verified the possible effects of brostallicin treatment on cells already overexpressing GSTpi and pre-incubated with zebularine.

In order to further characterize LNCaP-GST cells, their sensitivity to cDDP was assessed. GSTpi overexpression resulted, as expected, in a slight decrease in cDDP efficacy. Thus, in 5-10 μ M range of cDDP concentrations, survival versus controls values were 32% in wt cells to 52% in LNCaP-GST. Increased resistance was also evident, although to a lesser extent, with the other cDDP concentrations used (fig.4.1.7,A).

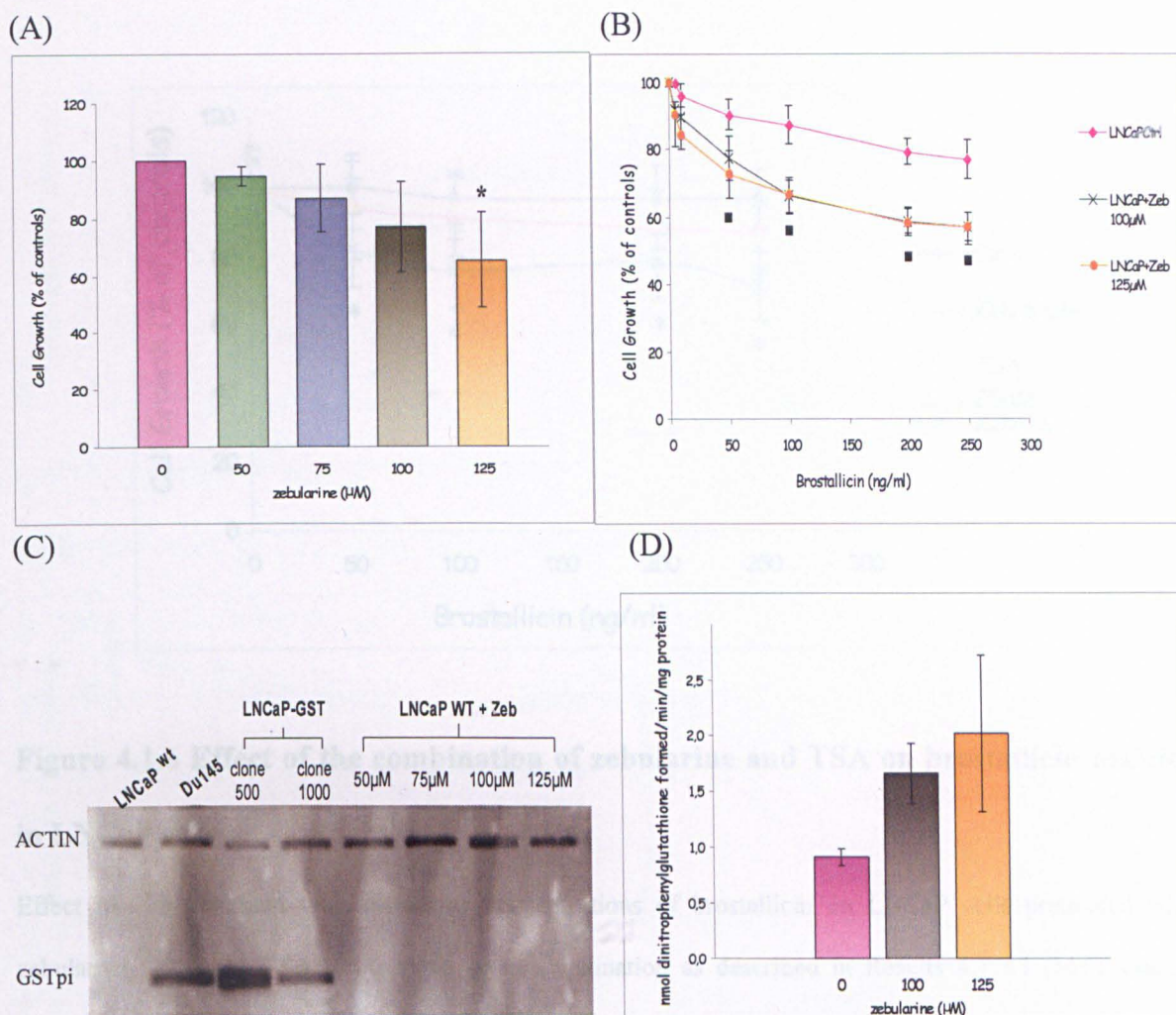


Figure 4.1.5 Effects of 96h treatment with zebularine on LNCaP cells

- (A) Effect of increasing concentrations of zebularine (96h treatment) on LNCaP cells, as determined by MTT assay. Growth inhibition by 125 μM zebularine was significantly different versus controls (*, $p < 0.05$, ANOVA test). Bars, S.D.
- (B) Effect of 72h treatment with increasing concentrations of brostallicin on LNCaP cells pretreated with zebularine for 96h, as determined by MTT assay. The graph is the mean of four independent experiments, each consisting of six replicates per group. Differences between treated groups versus controls are statistically different, according to the ANOVA test (\blacksquare , $p < 0.01$ at each brostallicin concentration, starting from brostallicin 50 ng/ml). Bars, S.D.
- (C) Western blotting against GSTpi on total proteins extracted by LNCaP treated or not with zebularine. LNCaP-GST clone 500 and clone 1000 are clones obtained by transfecting LNCaP with GSTpi cDNA and used as positive controls. Actin was used as housekeeping gene to detect the homogenous gel loading.
- (D) Total GST activity as determined by CDNB assay. The histogram is the mean of three independent experiments. Differences are not statistically significant. Bars, S.E.

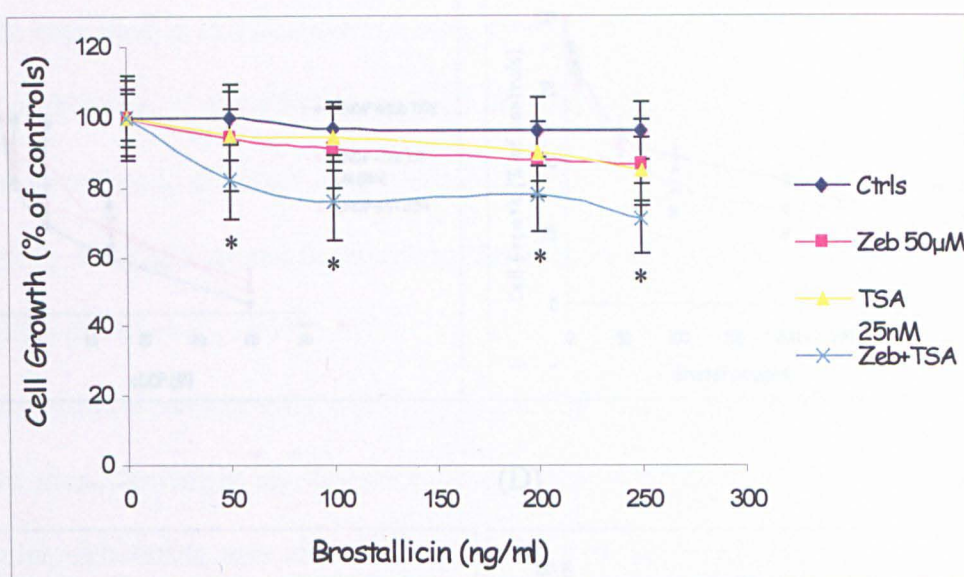


Figure 4.1.6 Effect of the combination of zebularine and TSA on brostallicin toxicity in LNCaP cells

Effect of 72h treatment with increasing concentrations of brostallicin on LNCaP cells pretreated with zebularine (72h) and TSA (24h) alone or in combination as described in Results 4.1.2.3 (MTT assay). Differences between (TSA+zebularine) group versus control group are statistically significant according to the ANOVA test (*, $p < 0.05$). Bars, S.D.

Pre-treatment of LNCaP wt cells with zebularine did not significantly affect their sensitivity to cDDP.

In LNCaP-GST cells the efficacy of brostallicin was slightly increased when cells were pretreated with zebularine at 50µM for 8 days (fig.4.1.7, panel B), or at 100µM for 96h (fig.4.1.7, panel C) before brostallicin (i.e. the same schedule adopted in LNCaPwt cells).

On the contrary, zebularine had completely no effect on cytotoxicity of brostallicin on Du145 cells (fig.4.1.7, D).

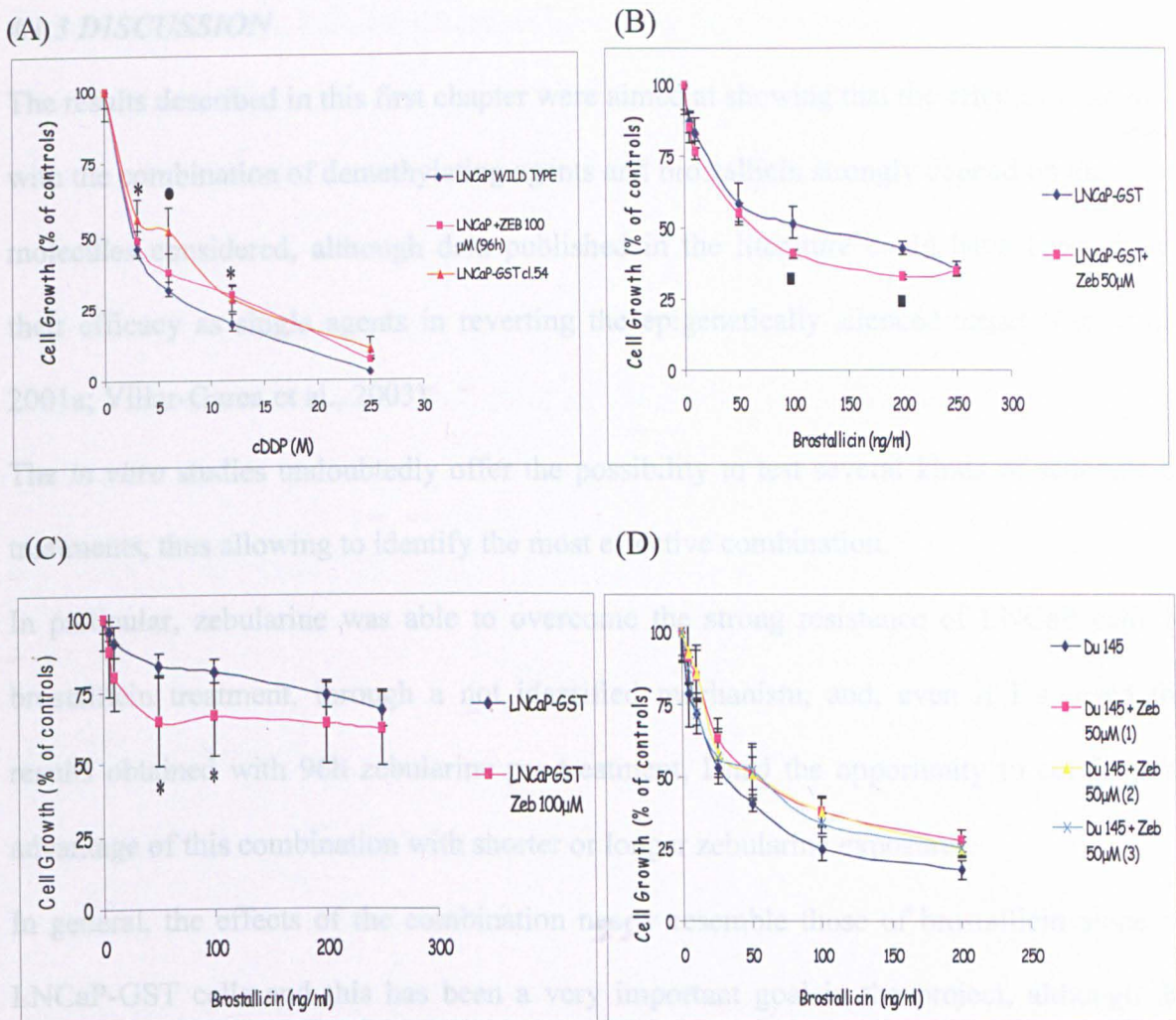


Figure 4.1.7 Effects of combination of zebularine and cDDP or brostallicin on GST expressing cells

(A) Effect of cDDP treatment on LNCaP-GST cells (clone 54) compared to LNCaPwt cells treated or not with zebularine, as determined by MTT assay; differences result to be statistically significant between LNCaPwt and LNCaP-GST cells treated with 3 μ M (*, $p < 0.05$), 6 μ M (●, $p < 0.001$) and 12 μ M (*, $p < 0.05$) cisplatin, according to the ANOVA test. Differences between LNCaPwt and LNCaP+Zeb100 μ M are not statistically significant at each cDDP concentration used.

(B) Effect of zebularine pretreatment (8 days) on LNCaP-GST cell sensitivity to brostallicin; at 100 and 200 ng/ml brostallicin, differences are statistically significant, with $p < 0.01$ (■) according to the ANOVA test. (C) Effect of zebularine pretreatment (96h) on LNCaP-GST cell or Du145 cell (D) sensitivity to brostallicin. In (C) differences are statistically significant (*, $p < 0.05$) at 50 ng/ml and 100 ng/ml brostallicin, according to the ANOVA test, in (D) no statistically significant differences were found at each brostallicin concentrations used. In all cases, MTT assay was performed, each group consisting of six replicates. Brostallicin and cDDP treatments took 72h.

4.1.3 DISCUSSION

The results described in this first chapter were aimed at showing that the effects obtained with the combination of demethylating agents and brostallicin strongly depend on the molecules considered, although data published in the literature could have been shown their efficacy as single agents in reverting the epigenetically silenced target (Lin et al., 2001a; Villar-Garea et al., 2003).

The *in vitro* studies undoubtedly offer the possibility to test several kinds of schedule of treatments, thus allowing to identify the most effective combination.

In particular, zebularine was able to overcome the strong resistance of LNCaP cells to brostallicin treatment, through a not identified mechanism, and, even if I showed the results obtained with 96h zebularine pre-treatment, I had the opportunity to confirm the advantage of this combination with shorter or longer zebularine exposures.

In general, the effects of the combination nearly resemble those of brostallicin alone on LNCaP-GST cells and this has been a very important goal in the project, although the specific re-expression of GSTpi has not been demonstrated. It cannot be neglected that there is an overall increase of the GST total enzymatic activity, that could explain the increased cytotoxicity of brostallicin. However, it is important to consider that the demethylating drugs do not specifically target an epigenetically silenced gene, but rather they exert a general activity on the genome, so it cannot be excluded that other genes could be affected by zebularine treatment, thus “helping” the brostallicin cytotoxicity; this could be suggested by its slight increase on LNCaP-GST cells pretreated with zebularine.

The zebularine- brostallicin combination does not prevent the possibility of treatment with other cytotoxic drugs (such as cisplatin) which are usually detoxified by GST/GSH system, and this widens the therapeutic strategy against prostatic tumours.

In summary all the experiments described gave me the opportunity to identify the best demethylating drug to combine with brostallicin: this goal could not be surely reached with

direct *in vivo* studies, both for ethical and economic reasons, because of the great number of animals needed.

CHAPTER 4.2

**IN VIVO COMBINATION OF THE DEMETHYLATING DRUG
ZEBULARINE AND THE MINOR GROOVE BINDER
BROSTALLICIN**

4.2.1 INTRODUCTION

The capability of zebularine to increase the *in vitro* antitumour activity of brostallicin prompted me to test this combination in an *in vivo* model.

In vivo studies are fundamental for testing of drugs, before evaluating their efficacy and safety in patients. In fact *in vivo* models allow not only the efficacy (i.e. effect on the tumour) of drugs or combinations to be evaluated, but also their toxicity (i.e. effect on normal tissues), although it is not practical to assess all possible schedules and concentrations used in the *in vitro* models. Moreover an *in vivo* system could change the *in vitro* activity of a drug, because of various mechanisms of detoxification and metabolism that may act within an organism.

Few *in vivo* studies have analyzed the efficacy of zebularine as demethylating agent in solid tumours although it has been demonstrated to be able to revert p16 promoter methylation in a model of bladder cancer (Cheng et al., 2004a). The re-expression of this tumour suppressor gene was achieved through daily treatment (18 days) with high doses of zebularine (500mg/kg and 1000mg/kg, i.p. and *per os*), that did not show significant toxicity in balb-c nude mice. Based on this study, I planned to pre-treat mice with a similar schedule of zebularine, before administering brostallicin.

4.2.2 RESULTS

4.2.2.1 Antitumour activity of zebularine-brostallicin combination in LNCaP xenografts in SCID mice

The choice of this immunodeficient strain of mice (instead of the usually used less immunodeficient nude mice) depended on the weak tumorigenicity of LNCaP cells used as xenograft model. The first objective of this study was to evaluate the toxicity of daily administration of 500mg/kg or 1000mg/kg zebularine in SCID mice, a suitable host for the

growth of LNCaP cells as xenografts. Firstly untransplanted mice were treated daily with zebularine i.p. and the body weights recorded. Zebularine 1000mg/kg was extremely toxic in SCID mice, starting from day 5 of administration (fig.4.2.1). This contrasts with the roughly total absence of toxicity recorded for balb-c mice (Cheng et al., 2003). For this reason the zebularine dose was reduced to 750mg/kg, but this did not prevent a significant loss of body weight (>25%) by day 10 after beginning treatment, although no deaths occurred.

In comparison, 500mg/kg zebularine was relatively well tolerated by these mice thus, although roughly 10% of weight loss could be observed, weight was rapidly regained after stopping treatment. Based on these results, zebularine at 500mg/kg was used in combination with brostallicin for LNCaP xenografted mice.

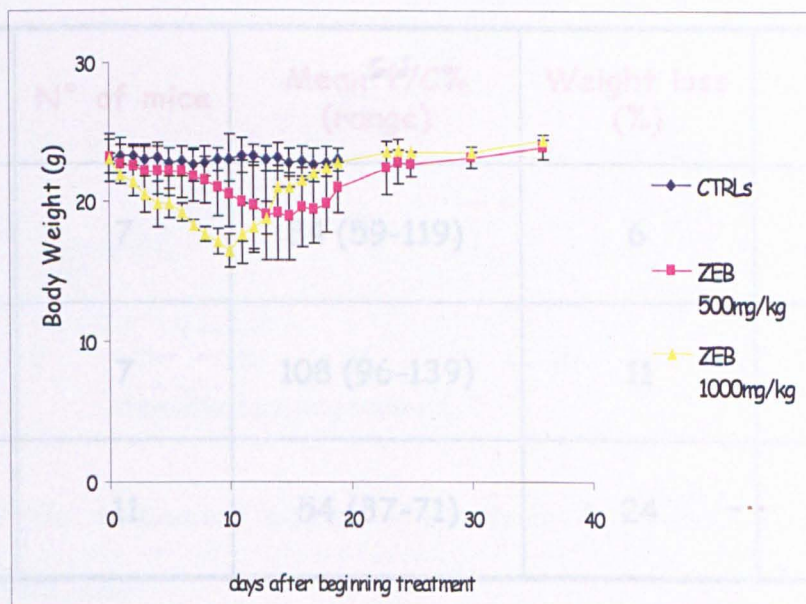


Figure 4.2.1 Toxicity of zebularine in untransplanted SCID mice

Mice were treated with zebularine i.p. once a day for 18 days. The body weight was recorded daily. Each group was of 5 mice. Day 0= first treatment Bars S.D.

The proposed schedule was that mice would to be treated for 18 days with zebularine and every 6 days with i.v. brostallicin at 0.4mg/kg for three administrations. But after the first

combination treatment followed by 2 treatments with zebularine, mice began to lose weight (fig.4.2.2, A) and therefore it was necessary to stop treatment. After a 6 day break for both drugs (during which body weight was partially restored), the schedule of the combination was resumed.

Brostallicin or zebularine alone did not show any substantial antitumour activity, as expressed by the mean T/C% values respectively of 108 and 84 (table 4.2.1, fig. 4.2.2,B). In contrast, the combination of the two drugs showed a mean T/C% value of 54, thus demonstrating that the zebularine-brostallicin combination had antitumour activity. As regards the toxicity (table 4.2.1), brostallicin and zebularine alone were well tolerated but for the combination, a 24% weight loss was recorded on the day of its nadir. This was partially restored by the end of the experiment, three weeks later.

| Treatment | N° of mice | Mean T/C% (range) | Weight loss (%) | Toxic deaths |
|--------------|------------|----------------------|--------------------|-----------------|
| Zebularine | 7 | 84 (59-119) | 6 | 0/7 |
| Brostallicin | 7 | 108 (96-139) | 11 | 0/7 |
| Combination | 11 | 54 (37-71) | 24 | 2/11 |

Table 4.2.1 Parameters related to the *in vivo* treatments of the zebularine-brostallicin combination on LNCaP tumours

Mean T/C% values calculated as described in Materials and Methods. In brackets are shown the highest and the lowest T/C% value reached one week after the last treatment. Toxicity expressed as toxic deaths (occurred on the 5th and 7th day after the first treatment with brostallicin respectively) and percentage of weight loss, calculated on the day of nadir, the fifth day after the last treatment with brostallicin.

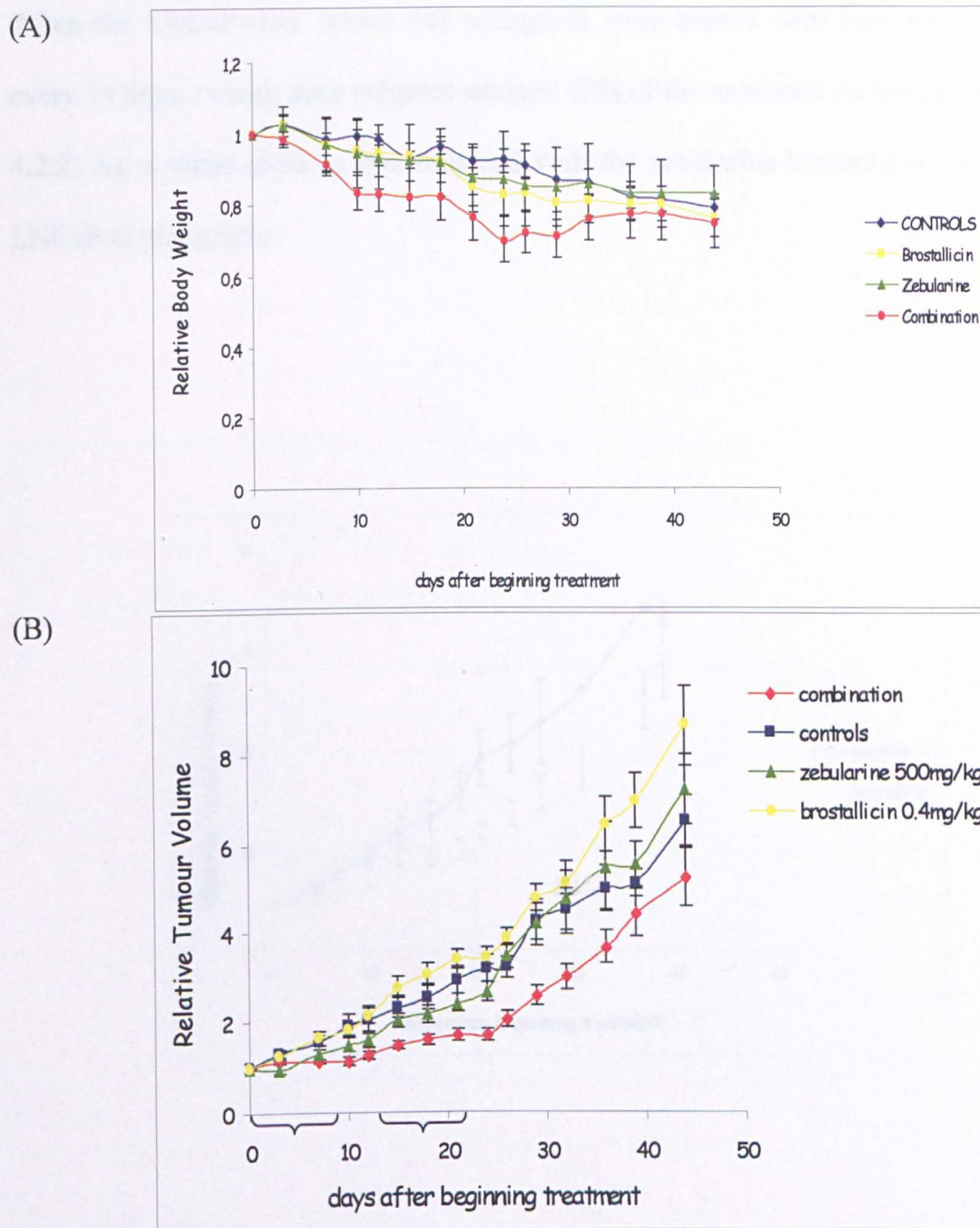


Figure 4.2.2 *In vivo* antitumour activity of zebularine-brostallicin combination on LNCaP transplanted mice

- (A) Relative body weight (calculated as described in Materials and Methods) curves. Body weight of each mouse was recorded on the same days of tumour measurements.
- (B) Tumour growth (expressed as relative tumour volume) curves of mice transplanted with LNCaP cells and treated with the combination zebularine-brostallicin. The horizontal brackets on X axis indicate the days of treatment. RTV values of the combination group are significantly (ANOVA test) reduced versus control group ($p < 0.01$), and versus brostallicin ($p < 0.0001$) and zebularine ($p < 0.05$) given alone, two days after the last treatment.

Day 0= first treatment with zebularine; bars S.E.

When the LNCaP-GST (clone 54) xenografts were treated with brostallicin (0.4mg/kg, every 14 days, twice), their volumes attained 62% of the untreated controls (fig.4.2.3, table 4.2.2, A), a value close to that obtained with the zebularine-brostallicin combination in LNCaPwt xenografts.

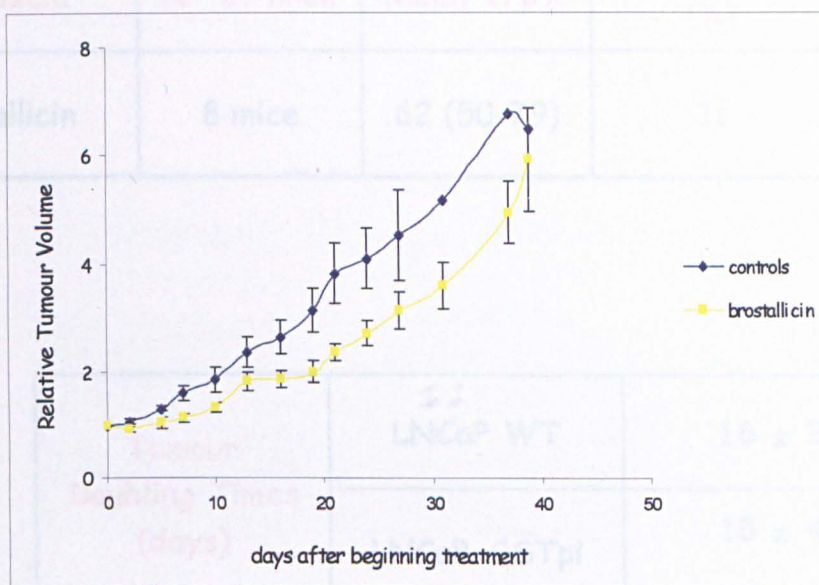


Figure 4.2.3 Antitumour activity of brostallicin on LNCaP-GSTpi tumours

Tumour growth curves of mice treated with brostallicin on day 6 and on day 20 (q14xd2), accordingly to the schedule of brostallicin treatment on LNCaPwt tumours.

RTV values of the treated group were significantly (ANOVA test) reduced versus the control group ($p < 0.05$), two days after the last treatment with brostallicin.

It is also important to note that the doubling time of LNCaP-GST tumours was not affected by the overexpression of GSTpi (table 4.2.2,B), because it has no role as tumour suppressor, unlike p16 in bladder cancer, whose re-expression resulted in the inhibition of tumour growth (Cheng et al., 2003).

(A)

| Treatment | N° of mice | Mean T/C% | Weight loss (%) | Toxic deaths |
|--------------|------------|------------|-----------------|--------------|
| Brostallicin | 8 mice | 62 (50-79) | 11 | 0/7 |

(B)

| Tumour Doubling Times (days) | LNCaP WT | 16 ± 2 |
|------------------------------|-------------|--------|
| | LNCaP-GSTpi | 15 ± 4 |

Table 4.2.2 LNCaP-GST tumour sensitivity to brostallicin

Mean T/C% values (A) and DT (B) values calculated as described in Materials and Methods. In brackets (A) the highest and the lowest T/C% values attained a week after the last treatment with brostallicin are indicated; the highest weight loss % value was attained a week after the last treatment with brostallicin.

4.2.2.2 Assessment of re-expression of GSTp in LNCaP xenografts after zebularine treatment

In order to establish if zebularine treatment affected the re-expression of GSTpi, which would correlate with the improvement of the antitumour activity of brostallicin, western blotting and immunohistochemistry were performed on tumour samples taken from mice sacrificed one day after the last treatment with zebularine. LNCaP tumour extracts did not show any expression of GSTpi (fig.4.2.4); although, it was very evident in LNCaP-GSTpi tumours used as controls.

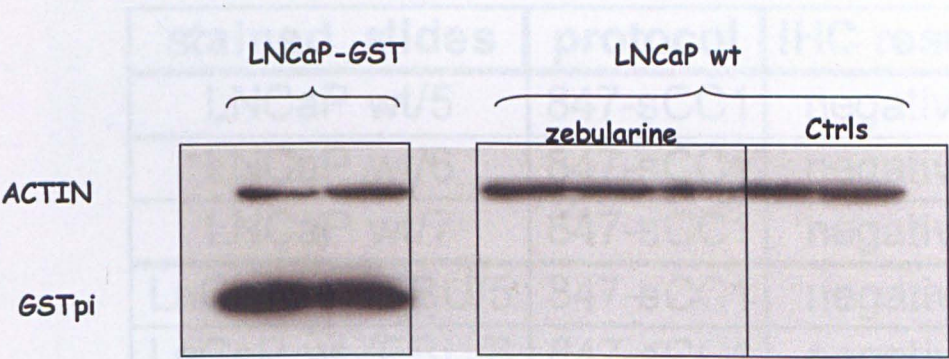


Figure 4.2.4 Western blotting for GSTpi expression in LNCaP tumour extracts

Detection of GSTpi expression on protein extracts from tumours of mice treated with two cycles of zebularine, excised one day after the last treatment, according to the schedule shown in fig.4.2.2,B. Proteins from LNCaP-GST tumours were used as positive controls. Actin was used to assess the homogeneity of gel loading.

Because this technique does not allow an appreciation of any effects produced at an individual cell level and because a weak re-expression could be masked by the total protein amount derived from the homogenized tumours, an immunohistochemical examination of tumours was carried out.

The morphology of LNCaP and LNCaP-GST tumours, evaluated by hematoxylin and eosin staining on fixed tumour slices, showed no detectable differences among tumour types (i.e. LNCaPwt and LNCaP-GST tumours). Focal necrosis (20-60% of the mass) and abundant hemorrhagic areas were seen within the tumour masses (fig 4.2.5, A).

As regards IHC, no positive GSTpi staining was detected in LNCaP tumours, treated or not with zebularine (table 4.2.3, fig 4.2.5, B). In contrast, a strong positivity was detected in LNCaP-GST tumours, and this was localized, as expected, in the nucleus and cytoplasm of the majority of tumour cells (fig.4.2.5, C).

| stained slides | protocol | IHC result |
|----------------|----------|------------|
| LNCaP wt/5 | 847-sCC1 | negative |
| LNCaP wt/6 | 847-sCC1 | negative |
| LNCaP wt/7 | 847-sCC1 | negative |
| LnCaP wtZEBU/5 | 847-sCC1 | negative |
| LnCaP wtZEBU/6 | 847-sCC1 | negative |
| LNCaPwtZEBU/7 | 847-sCC1 | negative |
| LNCaPwtZEBU/8 | 847-sCC1 | negative |
| LNCaPwtZEBU/9 | 847-sCC1 | negative |
| LNCaPwtZEBU/10 | 847-sCC1 | negative |
| LNCaP GST/6 | 847-sCC1 | >90% |
| LNCaP GST/7 | 847-sCC1 | >90% |
| LNCaP GST/8 | 847-sCC1 | >90% |

Table 4.2.3 Summary of IHC detection of GSTpi in LNCaP tumours

847-sCC1 is the IHC protocol followed by Ventana discovery autostainer.

Samples LNCaPwt ZEBU 5-7 derived from mice treated with one cycle of zebularine; samples LNCaPwtZEBU 8-10 derived from mice treated with two cycles of zebularine (as indicated in fig 4.2.2, B)

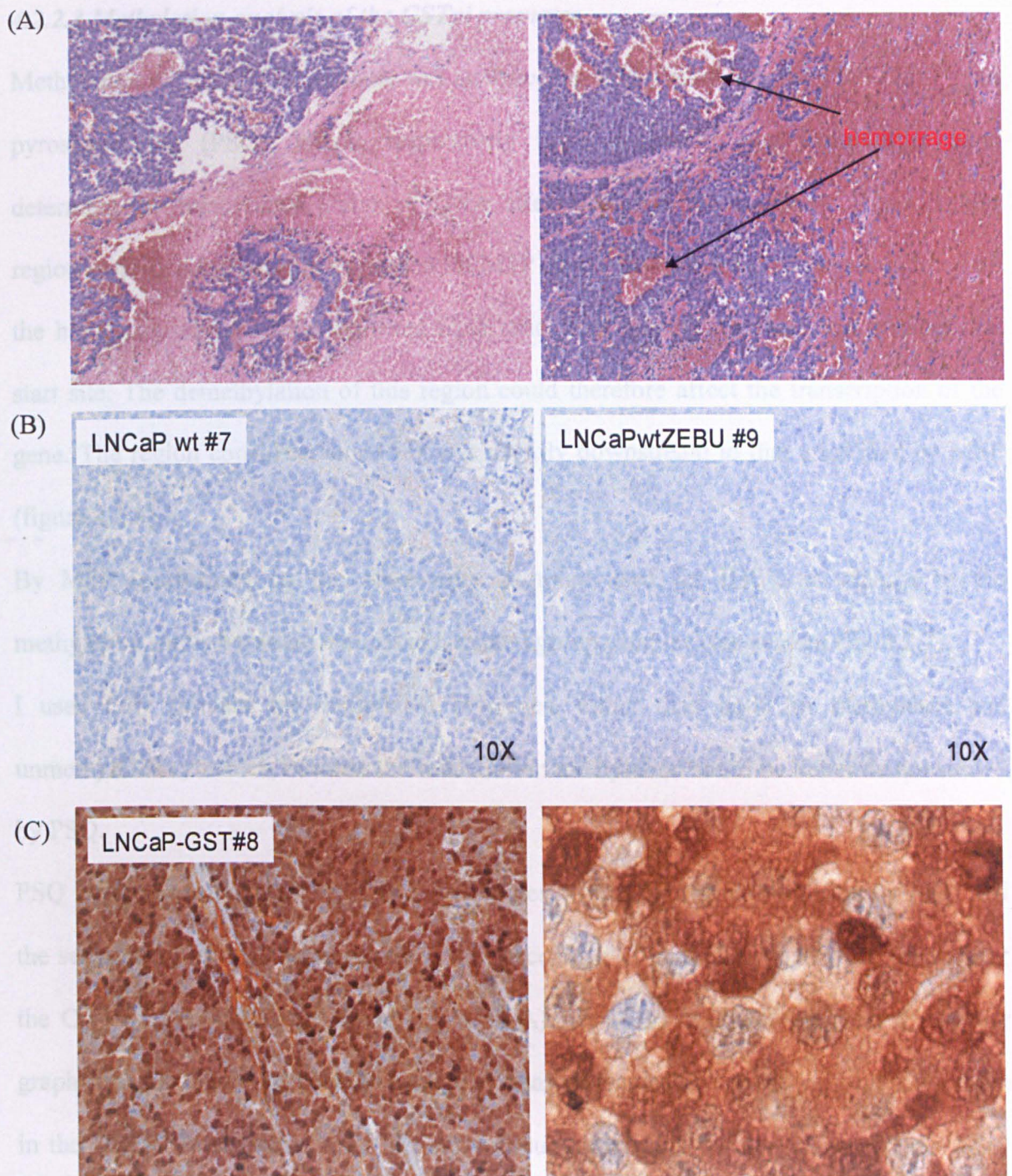


Figure 4.2.5 Morphology and GSTpi expression in LNCaP tumours by IHC

(A) Morphology of LNCaPwt (left) and LNCaP-GST (right) tumours evaluated by Haematoxylin and Eosin staining (Hemorrhagic areas are indicated).

(B) IHC analysis for GSTpi expression in LNCaPwt tumours treated (right) or not (left) with zebularine. Counterstaining was with hematoxylin.

(C) IHC analysis for GSTpi expression in LNCaP-GST tumours, showing the cytosolic and nuclear distribution of GSTpi. Counterstaining was with hematoxylin.

10x of original magnification; in (C, right) 40x of original magnification.

4.2.2.3 Methylation analysis of the GSTpi promoter

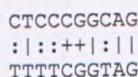
Methylation of the GSTP1 promoter was analyzed by methylation-specific PCR (MSP) and pyrosequencing (PSQ), which, respectively in a qualitative and quantitative way, determine the methylation status of DNA after bisulphite modification. The promoter region amplified by the primers chosen for MSP corresponds to the CpG island region with the highest observed versus expected CpG ratio, immediately upstream the transcription start site. The demethylation of this region could therefore affect the transcription of the gene. The region considered for PSQ was slightly downstream to that amplified by MSP (figure 4.2.6).

By MSP (performed on the same mice tumours used for IHC), no change in the methylation status between controls and treated group samples was evident (fig.4.2.7).

I used only primers for methylated cytosines, rather than both for methylated and unmethylated cytosines, because the reduction in methylation could be better demonstrated by PSQ.

PSQ was used to analyze 14 CpG dinucleotides within the region amplified by PCR and the sequencing of this region provided the percentage of methylated cytosines for each of the CpG dinucleotides considered (fig 4.2.8,A). As could be evaluated from the scatter graph showing the % methylation change of treated samples versus controls, no differences in the GSTP1 methylation level are evident, suggesting that the zebularine did not bring about the demethylation of the GSTP1 promoter (fig.4.2.8, B).

These data confirm, accurately and quantitatively, those obtained using MSP, and support the observation of the absence of GSTpi protein re-expression demonstrated by western blotting.



Top: CpG island region identified by Methprimer software (Li and Dahiya, 2002), accordingly to Gardiner and Frommer criteria (Gardiner-Garden and Frommer, 1987). The region with the highest Obs_{CpG}/Exp_{CpG} ratio is highlighted.

Symbols + indicate the CpG dinucleotides. Sequencing primer sequence for PSQ is highlighted in yellow.

GSTpi

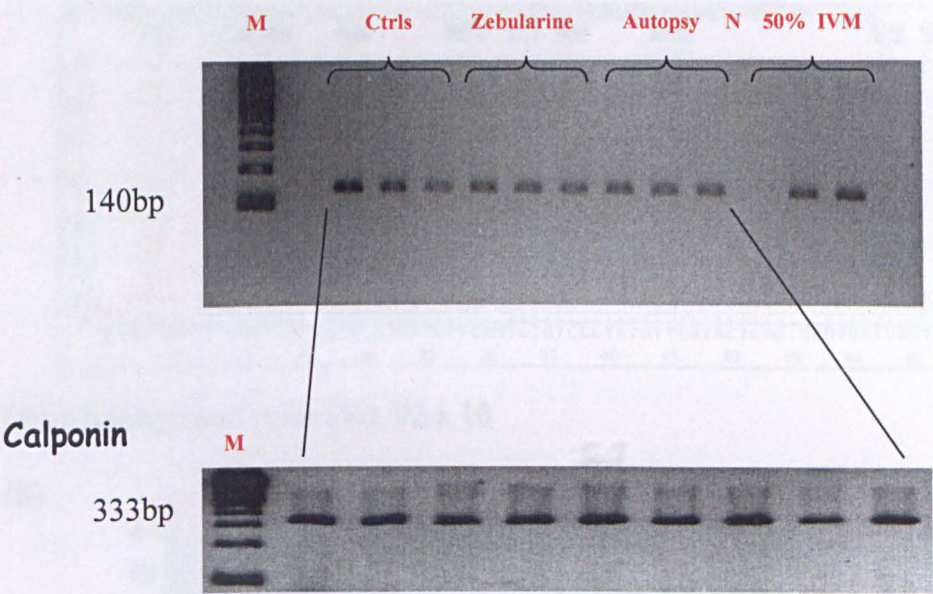
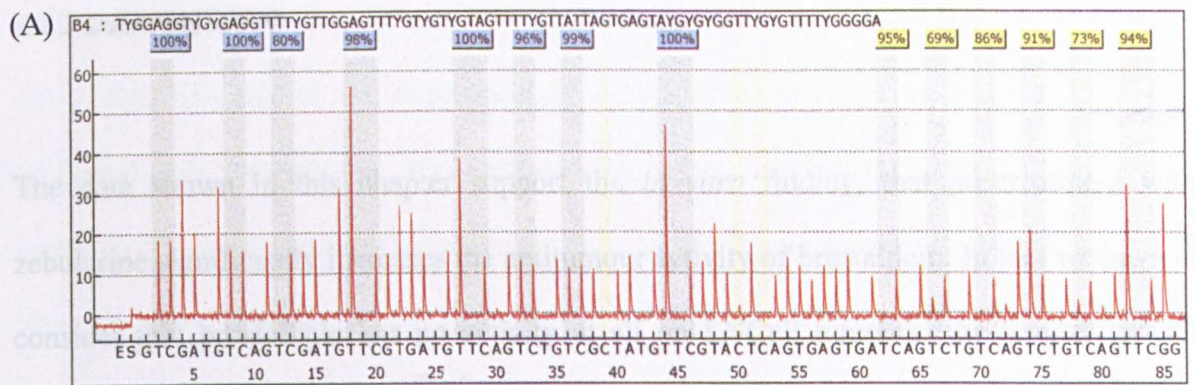


Figure 4.2.7 Agarose gel analysis of MSP of the GSTP1 promoter

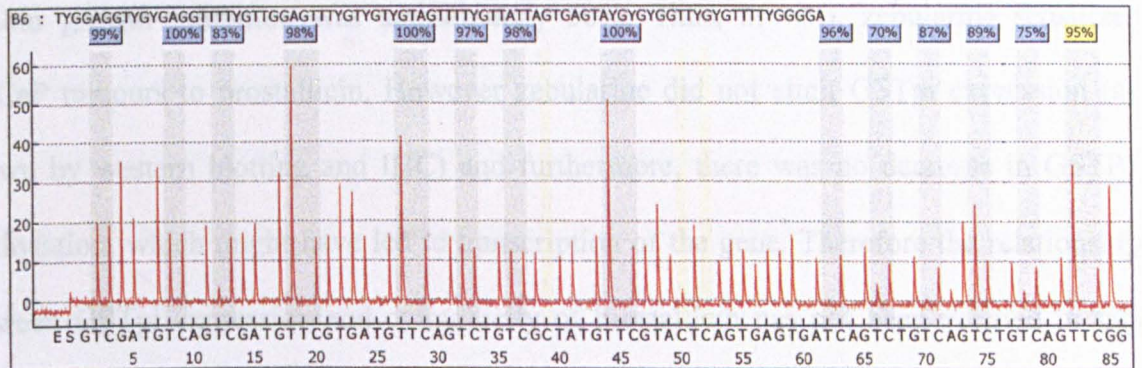
Primers for methylate cytosines were used. The IVM, 50% and N are controls with respectively 100%, 50% and 0% methylation (IVM, In Vitro Methylated, N, normal).

Calponin was used as control for homogeneous bisulfite modification.

Ctrlis indicate samples deriving from untreated mice, zebularine indicates samples taken one day after the last treatment with zebularine (corresponding to the same material analyzed by western blotting and IHC), and autopsy indicates samples taken from animals sacrificed at autopsy.



Mean Methylation value (%): 92 ± 11



Mean Methylation value (%): 92 ± 10

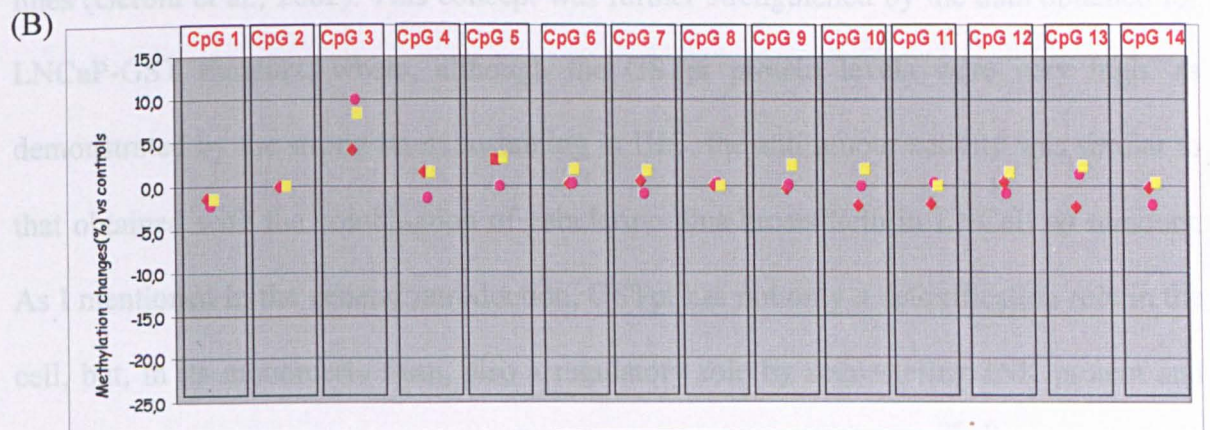


Figure 4.2.8 Pyrosequencing of the GSTP1 promoter

(A) Pyrograms of 14 CpG dinucleotides comprised in the region amplified by PCR and analyzed using PSQ primers (see figure 4.2.6). The highlighted values indicate the percentage of methylation in each position considered. The upper sequence represents the sequence analyzed, the lower sequence represents the dispensation order of nucleotides; Y axis represents arbitrary units related to the PPi reaction. The 14 CpG analyzed are 332 bp upstream of exon 1 of the gene. The upper graph represents the analysis of a representative LNCaP control tumour, while the lower graph represents the analysis of a representative LNCaP tumour treated with zebularine. (B) Methylation changes at each CpG site, calculated as ratio of the % methylation of zebularine treated samples versus the % methylation of controls at the same CpG dinucleotide. Each point represents a treated mouse.

4.2.3 DISCUSSION

The data shown in this chapter support the *in vitro* finding, that pre-treatment with zebularine significantly increases the antitumour activity of brostallicin. In fact we have to consider that brostallicin has no activity at all on LNCaP tumours (the tumour growth curves are superimposed to that of the controls), but when combined with zebularine, the tumour growth inhibition was approximately 50%. Thus, *in vivo*, zebularine sensitizes LNCaP tumours to brostallicin. However zebularine did not elicit GSTpi expression (as shown by western blotting and IHC) and furthermore, there was no decrease in GSTP1 methylation, which might have led to transcription of the gene. Therefore the relationship between GSTpi expression and cytotoxicity of brostallicin has not been verified in this model, even though it has been extensively studied and confirmed in other tumour cells lines (Geroni et al., 2002). This concept was further strengthened by the data obtained for LNCaP-GST tumours, where, although the GSTpi protein levels were very high, as demonstrated by the strong immunostaining in IHC, the antitumour activity was similar to that obtained with the combination of zebularine plus brostallicin in LNCaP wt tumours. As I mentioned in the general introduction, GSTpi has not only a detoxification role in the cell, but, in its monomeric form, also a regulatory role by sequestering JNK protein and preventing it exerting its apoptotic function on the cells in unstressed conditions. Thus we cannot exclude the possibility that very high GSTpi expression levels in LNCaP-GSTpi cells could have had the paradoxical effect of interfering with the apoptotic process, a means by which brostallicin can induce cell death.

At the moment, I have not experimentally demonstrated this possible alternative role of GSTpi in reducing brostallicin cytotoxicity, which thus remains speculative.

One hypothesis that seems to emerge from analyzing the *in vivo* data is that the enzymes involved in the intracellular activation of brostallicin in LNCaPwt (in combination) and LNCaP-GST may be different.

It was clear that LNCaP-GST cells are more sensitive to brostallicin than LNCaP cells and this is probably due to the expression of GSTpi. Thus the *in vitro* study showed that LNCaPwt cells not only have no detectable GSTpi protein expression, but also no activity on the CDNB substrate, which conversely became very high on the transfected cells.

However, zebularine was not effective in GSTP1 demethylation in LNCaP tumours, even though a sensitization to brostallicin was evident.

It must be considered that many other epigenetically silenced genes may have had their methylation reverted after treatment. The absence of antitumour activity of zebularine alone might suggest that tumour suppressors do not undergo such re-expression.

As described previously, the current perception is that demethylating agents have no a specific target of activity. However it is feasible that different levels of methylation in genes belonging to different pathways could determine a higher or lower sensitivity to being demethylated by treatments with these epigenetic drugs.

CHAPTER 4.3

INVOLVEMENT OF GST ENZYMES IN THE ANTITUMOUR ACTIVITY OF BROSTALLICIN

4.3.1 INTRODUCTION

I have above described the effects of the demethylating agent zebularine on the antitumour activity of brostallicin, which is clearly increased. This effect was expected to arise from the reversion of the methylation of the GSTP1 gene promoter, but this has been shown not to be the case.

As discussed in the previous chapter, the increased cytotoxic activity of brostallicin might, then, be explained by a non-specific effect of zebularine on some unidentified target. In my opinion, this does not detract from the results obtained, above all because I have shown activity in an *in vivo* system. Indeed it encourages further investigation of the possible consequences of zebularine treatment.

A suggestion came from the *in vitro* data showing that LNCaP-GSTpi cells were more sensitive to brostallicin if pre-treated with zebularine (fig.4.1.7, B and C). This was different to that observed in Du145 cells, whose sensitivity to brostallicin was not increased by zebularine (fig. 4.1.7, D). Moreover, another suggestion derived from the increased GST total activity detected by the CDNB assay, the results of which did not correspond to any signal evident by western blotting using an antibody recognizing specifically GSTpi: CDNB is a substrate for all classes of GST, so we cannot exclude the possibility that other promoter methylation silenced GST classes may have been reactivated by zebularine.

In this chapter I will investigate the possible involvement of these classes of GST enzyme in the increased antitumour activity of brostallicin after treatment with zebularine.

4.3.2 RESULTS

4.3.2.1 Role of different cytosolic classes of GST in the antitumour activity of brostallicin

GSTP1 methylation is not a peculiarity of the prostatic cancer cell line LNCaP alone, but has also been detected in the breast cancer cancer cell line MCF-7 (Jhaveri and Morrow, 1998) and this correlates with the lack of GSTpi protein expression in these cells. However, the very low GST total activity, as determined by the CDNB assay (fig.4.3.1, (Tew et al., 1996)), suggested that MCF-7 are deficient in this detoxification system and are probably unable to activate brostallicin.

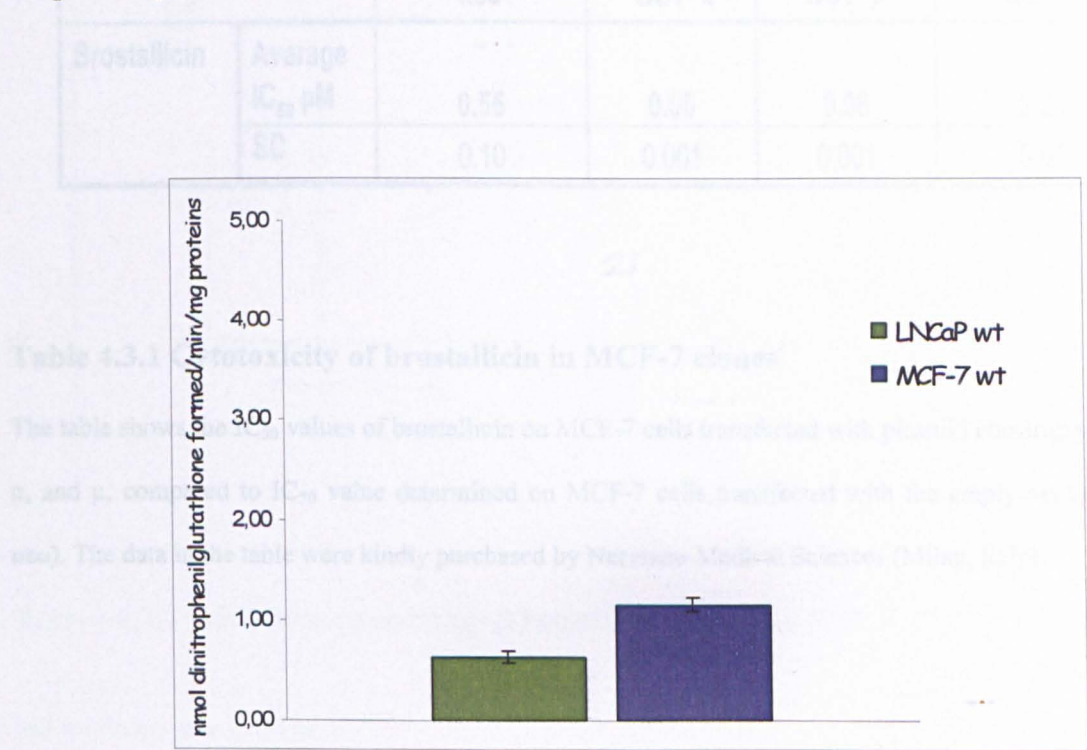


Figure 4.3.1 CDNB assay on LNCaP wt and MCF-7 wt cells

The CDNB assay was performed on cytosolic extracts of untreated cells, according to the method described. Bars, S.D.

Clones of MCF-7 cells transfected with plasmid constructs containing the cDNA encoding for GSTα, GSTμ and GSTπ classes were available at Nerviano Medical Sciences (Milan, Italy), where their sensitivity to brostallicin were determined in parallel.

The results summarized in table 2 show that both GSTpi and mu made MCF-7 ten times more susceptible to the cytotoxicity of brostallicin than the empty vector, while GSTα overexpressing cells are only two times more sensitive to brostallicin.

| | | Cells | | | |
|--------------|--------------------------------|--------------|-----------------|-----------------|----------------|
| | | MCF-7 neo | MCF-7 GST- π | MCF-7 GST- μ | MCF-7 GST-α |
| Brostallicin | Average IC ₅₀ μM | 0.55 | 0.06 | 0.06 | 0.24 |
| | SD | 0.10 | 0.001 | 0.001 | 0.01 |

Table 4.3.1 Cytotoxicity of brostallicin in MCF-7 clones

The table shows the IC₅₀ values of brostallicin on MCF-7 cells transfected with plasmid constructs of GST π, α, and μ, compared to IC₅₀ value determined on MCF-7 cells transfected with the empty vector (MCF-7/neo). The data in the table were kindly purchased by Nerviano Medical Sciences (Milan, Italy).

4.3.2.2 Affinity studies of brostallicin for classes μ and π of GST

Kinetic studies of the reaction between brostallicin and GSTmu and GSTpi were performed by Prof. Caccuri’s group at the University of Tor Vergata in Rome.

The results show that GSH is required for brostallicin binding to GSTpi protein and that brostallicin behaves as an uncompetitive inhibitor of GSH (fig.4.3.2,A). Moreover, brostallicin behaves as a non-competitive inhibitor of the co-substrate CDNB (fig. 4.3.2,C). Both inhibition constants were in the micromolar range with Ki values of about 40μM (fig.4.3.2, B and D).

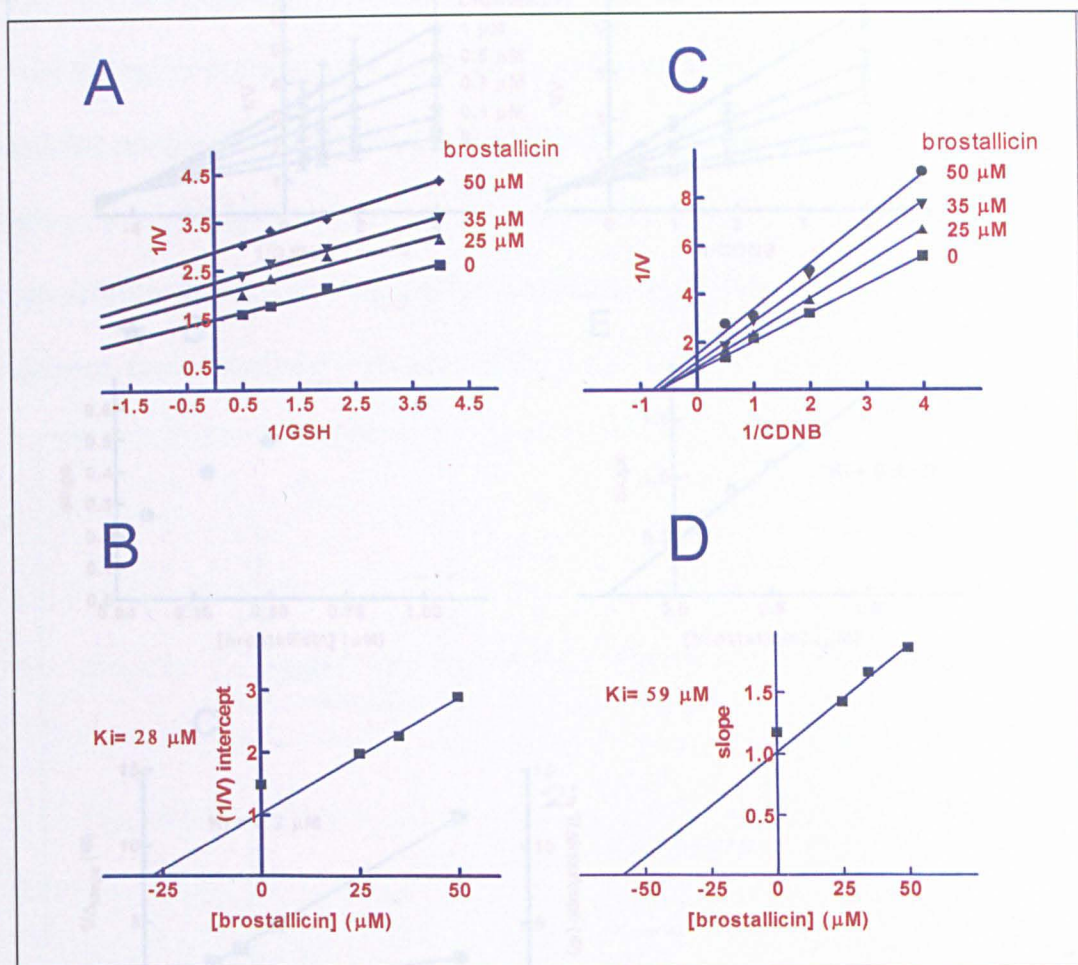


Figure 4.3.2 Inhibition properties of brostallicin towards GSTpi

Figure 4.3.3 Inhibition properties of brostallicin towards GST μ

See the description in the text.

See the description in the text.

When GST μ was considered (fig.4.3.3), brostallicin behaved like a mixed-type inhibitor in respect to both GSH and CDNB (fig.4.3.3, A and D) and the K_i values were about 1 μM (the highest value was 1.6 μM and the lowest 0.3 μM , fig. 4.3.3, E), about 40 times lower than K_i found for GSTpi.

These results suggest that GST μ has a binding site for brostallicin, that has higher affinity than that of GSTpi.

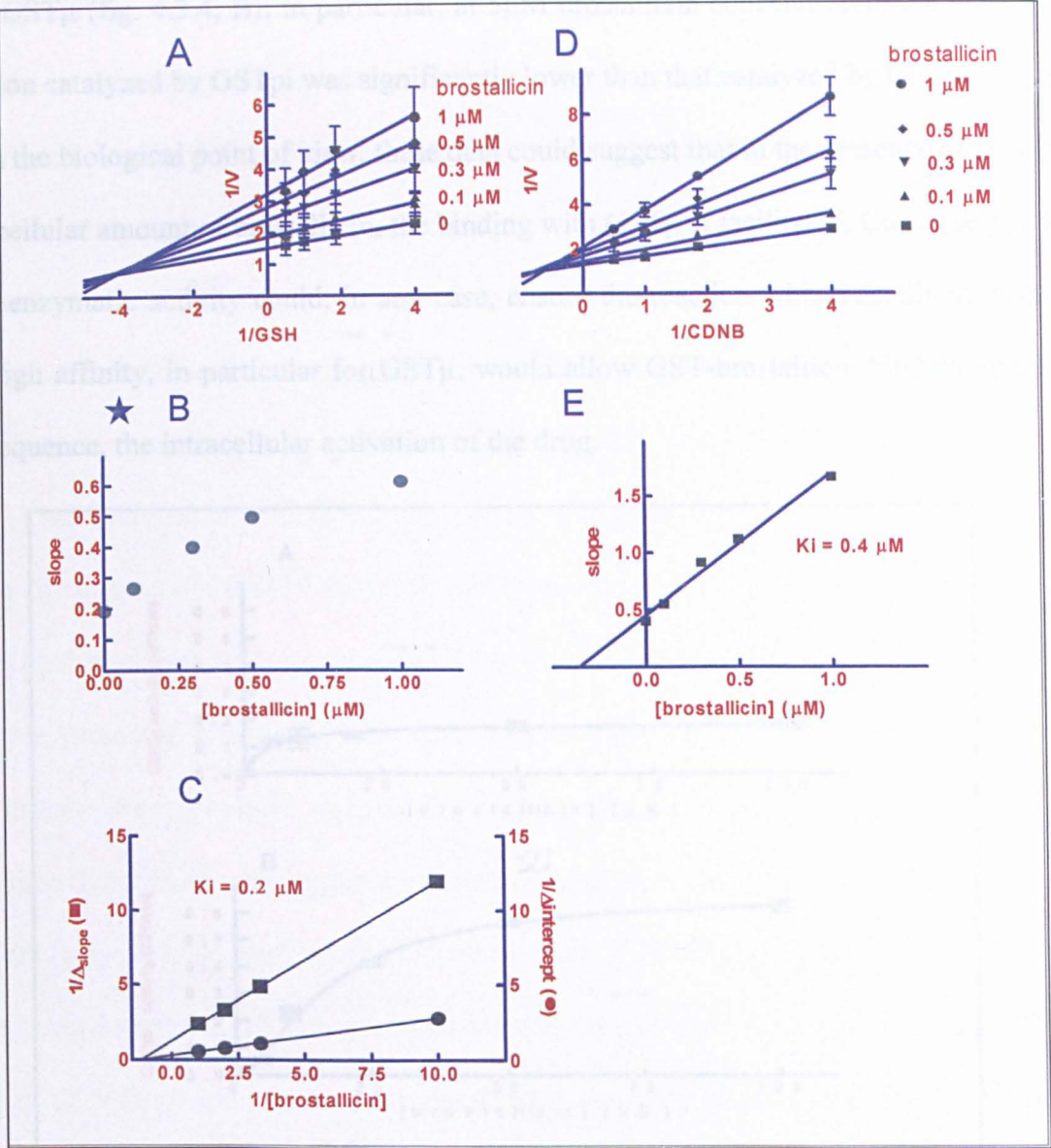


Figure 4.3.3 Inhibition properties of brostallicin towards GST μ

See the description in the text.

When the effects of brostallicin concentration on the initial velocity of reaction catalyzed by GST μ and pi were studied (fig.4.3.4), it was evident that the GST μ reached maximum velocity at 5 μM brostallicin (fig.4.3.4,A) and this depended on the high affinity of GST μ for brostallicin. The GSTpi kinetics were characterized by a slow reaction rate at low brostallicin concentrations, followed by an exponential rate increase at higher concentrations up to a maximum velocity which was four times higher than that observed

with GST μ (fig. 4.3.4, B): in particular, at 5 μ M brostallicin concentration, the velocity of reaction catalyzed by GST π was significantly lower than that catalyzed by GST μ . From the biological point of view, these data could suggest that in the presence of very low intracellular amount of brostallicin, the binding with GST μ is facilitated. Conversely, a low GST enzymatic activity could, in any case, ensure the reaction with brostallicin, because the high affinity, in particular for GST μ , would allow GST-brostallicin binding and, as a consequence, the intracellular activation of the drug.

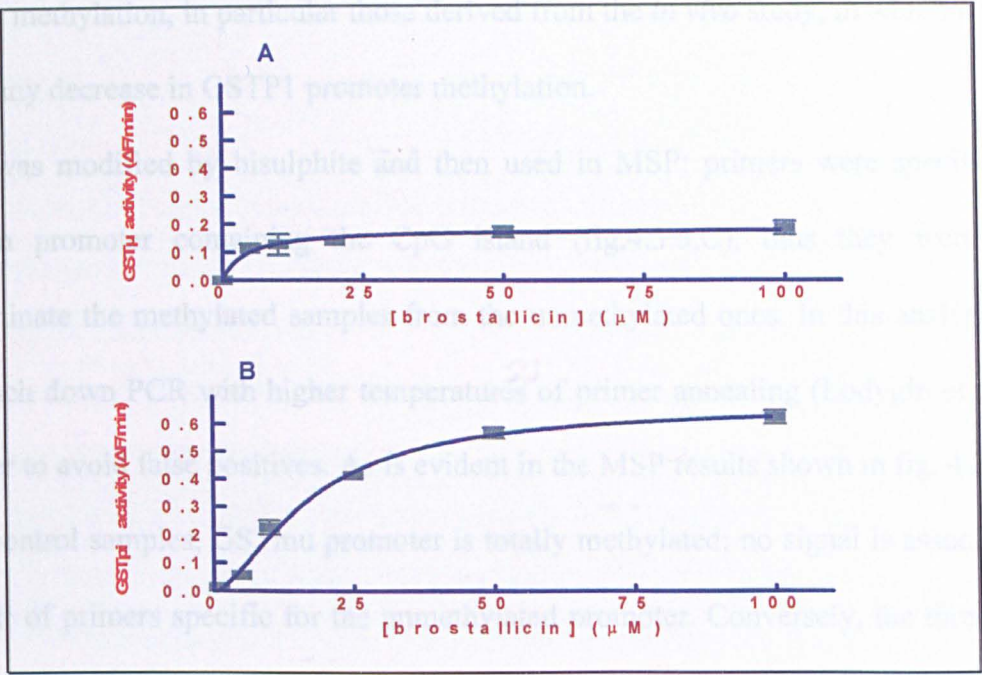


Figure 4.3.4 Effects of brostallicin concentration on the velocity of reaction with GST enzymes

Values obtained using increasing concentrations of brostallicin were plotted against GST μ (upper graph) or GST π (lower graph) activity (determined as described in Materials and Methods) expressed as Δ fluorescence (F) recorded/ minute.

4.3.2.3. GSTmu methylation analysis in LNCaP tumours

The above data suggests that brostallicin can be activated intracellularly not only by GSTpi, but also by GSTmu and that the higher affinity of this latter class for brostallicin can more easily allow its activation when low levels of the enzyme are present.

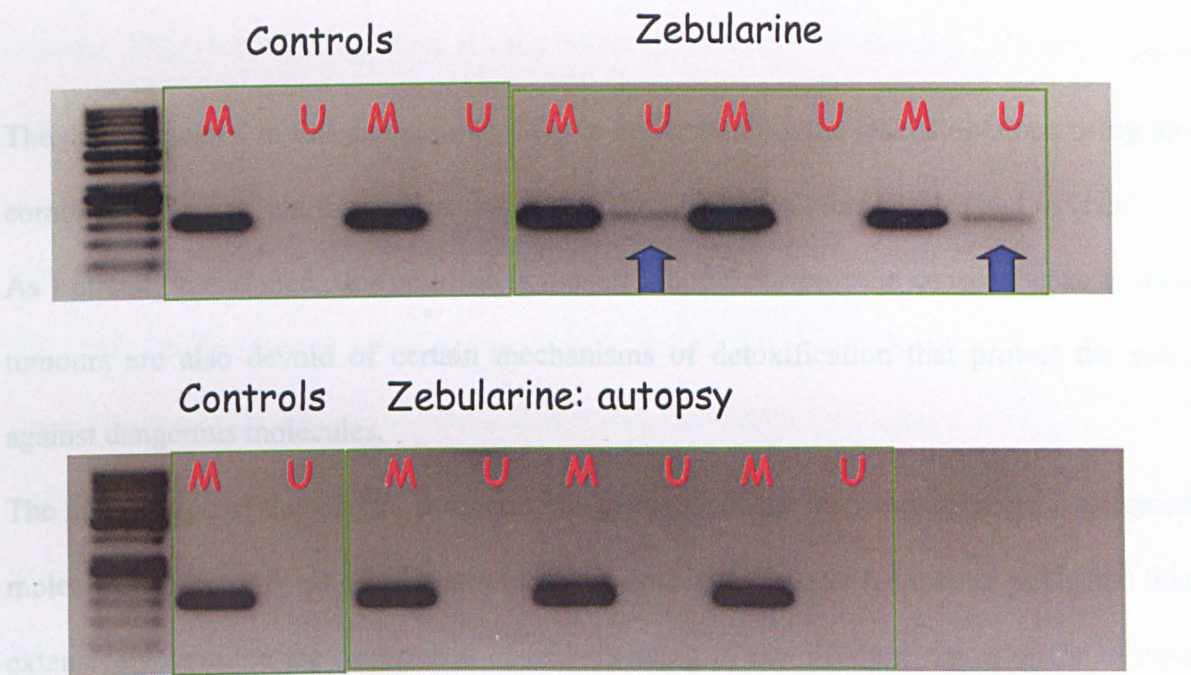
Figure 4.3.5 shows that the GSTmu promoter contains a CpG island and, as a consequence, the same mechanism of silencing characterizing GSTP1 gene could be relevant to GSTM1. Therefore, I examined the methylation status of the samples previously analyzed for GSTP1 methylation, in particular those derived from the *in vivo* study, in which I could not detect any decrease in GSTP1 promoter methylation.

DNA was modified by bisulphite and then used in MSP: primers were specific for the GSTmu promoter containing the CpG island (fig.4.3.5,C), thus they were able to discriminate the methylated samples from the unmethylated ones. In this analysis, I used the touch down PCR with higher temperatures of primer annealing (Lodygin et al., 2005) in order to avoid false positives. As is evident in the MSP results shown in fig. 4.3.6, in the three control samples, GSTmu promoter is totally methylated: no signal is associated with the pair of primers specific for the unmethylated promoter. Conversely, the three samples taken from mice treated with zebularine for two cycles (when the effects of zebularine on the antitumour activity of brostallicin were more evident) show a detectable signal corresponding to promoter demethylation in 2 of the 3 mice used in the analysis.

This demethylation was reverted in samples taken from mice at autopsy.

So it seems that zebularine treatment had no effect on GSTP1 promoter methylation, but it demethylated at least partially the GSTmu promoter. Comparing the GSTP1 CpG island (fig. 4.2.6) and the GSTM1 CpG island (fig. 4.3.5, C), a lower density of CpG dinucleotides is evident in the latter, so I speculate that it could be easier to induce demethylation in the GSTM1 promoter than in the GSTP1 promoter by treating mice with zebularine.

(A) GSTM1



(B) Calponin

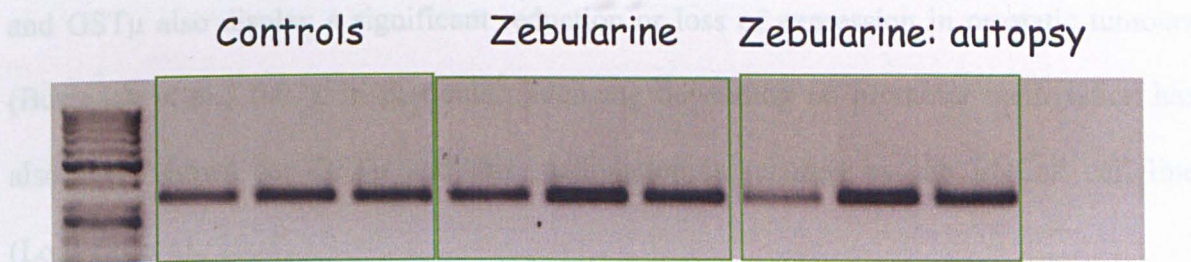


Figure 4.3.6 MSP on bisulfite modified GSTM1 promoter

(A) Primers for methylate (M) and unmethylated (U) cytosines were used.

“Controls” indicate samples deriving from untreated mice, “zebularine” samples were taken one day after the last treatment with zebularine (corresponding to the same fragments analyzed by western blotting, IHC and GSTP1 methylation), and “autopsy” samples were taken from sacrificed animals at autopsy. The arrows highlight the detection of unmethylated cytosines in samples treated from the zebularine group.

Calponin (B) was used as control for homogeneous bisulfite modification of the samples analyzed for GSTM1 methylation.

4.3.3. DISCUSSION

The data presented in this chapter may help in understanding the results obtained using the combinations of zebularine and brostallicin on the LNCaP cell line *in vitro* and *in vivo*.

As I already mentioned, this cell line is a useful model for prostate cancer, because such tumours are also devoid of certain mechanisms of detoxification that protect the cells against dangerous molecules.

The methylation of the GSTP1 promoter has been one of the most extensively investigated molecular characteristics of prostatic tumours, and many papers have been published that extensively examine the occurrence of this silencing in the different stages of the disease (Nelson et al., 2003; Perry et al., 2006; Meiers et al., 2007). Very recently, there has been a shift towards the analysis of all classes of the GST family and this has revealed that GST α and GST μ also display a significant reduction or loss of expression in prostatic tumours (Bostwick et al., 2007). In particular, silencing depending on promoter methylation has also been shown for GST μ and this methylation is retained by the LNCaP cell line (Lodygin et al., 2005).

This sheds new light on the data obtained, both *in vitro* and *in vivo*, with the combination of the demethylating agent zebularine and brostallicin.

The data obtained for the transfected MCF-7 cells and the kinetic studies have demonstrated the ability of GST μ to catalyze the intracellular activation of brostallicin. Furthermore, at low concentrations, GST μ has shown a higher affinity for this drug than GST π , suggesting that it could be “easier” to capture brostallicin when there are low concentrations of both enzyme and drug in the cell.

This is consistent with the mechanism related to methylation reversion by demethylating drugs: these do not totally eliminate methyl groups from DNA: the level of demethylation depends (among other possible factors) on the percentage of methylated cytosines in the CpG island spanning the promoter analyzed. This could explain the different susceptibility

of different genes to treatment with demethylating drugs, as is clearly the case for GSTpi and mu. However their function is only temporarily restricted and so, differently from silencing that depends on a mutation or a chromosomal aberration, the decreased methylation may be reverted after stopping demethylating agent treatment, with kinetics that may also be specific for each gene.

This may be the case for GST μ because no unmethylated signals were evident in the samples taken at autopsy, three weeks after the last treatment with zebularine.

These data altogether indicate that zebularine is able to potentiate brostallicin antitumour activity through its demethylating activity not on GSTpi, as was originally hypothesised, but GSTmu. Admittedly, the level of demethylation was marginal, but I have also argued that even if this resulted in very small increases in GSTmu activity (which has yet to be demonstrated), because of the affinity of GSTmu for brostallicin, it may have been sufficient to cause the *in vivo* (and *in vitro*) sensitization.

Essentially, the hypothesis generated, based on the mechanism of action of brostallicin, were therefore confirmed *in vitro* and *in vivo*.

CHAPTER 4.4

METHYLATION ANALYSIS OF THE MAGE 1A GENE IN A PHASE I STUDY OF DECITABINE IN PATIENTS RECEIVING EPIRUBICIN, CISPLATIN AND 5-FU FOR ADVANCED GASTRIC OR OESOPHAGEAL CANCER

4.4.1 INTRODUCTION

Following the discovery of the fundamental role of epigenetic aberrations in cancer development, new therapeutic prospects have arisen for several kind of cancer. This possibility has been further supported by the FDA approval of decitabine for the treatment of haematopoietic malignancies and many clinical studies have been designed to widen its application also to solid tumours. But in contrast to the encouraging results obtained for the blood cancers, no significant results have been obtained by treating solid tumours with decitabine as single agent. Nevertheless, as I mentioned in the general introduction, preclinical data have suggested the possibility of combining demethylating molecules with cytotoxic drugs, especially when the acquisition of drug resistance is related to the promoter methylation of genes involved in cellular responses to chemotherapy (Plumb et al., 2000).

In order to establish the doses and the schedule of decitabine treatment that is able to induce DNA demethylation, a phase I clinical study has been conducted in patients affected by gastro-oesophageal tumours and receiving epirubicin, cisplatin and protracted venous infusional 5-FU (ECF) as standard treatment.

Gastro-oesophageal tumours are characterized by the epigenetic inactivation of several tumour suppressor genes and of genes involved in cell adhesion (CDH1), DNA repair (MGMT, GSTP1) and cell cycle control. Reversal of hypermethylation by treatment with decitabine may allow the reactivation of these genes and, hence, the possibility of circumventing drug resistance in cancer cell.

Many studies have shown that low doses of decitabine are more effective than higher doses in inducing DNA hypomethylation with minimal toxicity. The first clinical studies attained this objective by a daily intravenous infusion of the drug (Schwartzmann et al., 2000). Recently, a phase I study on solid tumours treated with decitabine in combination with carboplatin has shown that a single dose of decitabine, administrated before carboplatin,

can be enough to obtain the highest level of demethylation in tumours about 10 days after treatment for each dose used and that this demethylation is reverted by day 22 (Appleton et al., 2007).

In this chapter I will describe the results of methylation analysis performed on tissue and blood taken 11 days after one decitabine treatment of patients with gastro-oesophageal cancer. The analyses have been carried out on the MAGE 1A gene as marker of the effect of the demethylating treatment, because, as I mentioned in the general introduction, this gene is only expressed in the testis and is bi-allelically methylated and transcriptionally silenced in normal tissues.

4.4.2 RESULTS

4.4.2.1 Methylation analysis of MAGE 1A gene

The methylation analysis was conducted on tissue and blood samples from seven patients from those recruited to the clinical study. Biopsies of normal mucosa and of primary tumour sites have been taken at the time of diagnosis (day 0), together with peripheral blood samples, and the methylation level established at this time point represented the reference level for the analysis conducted on samples taken 11 days after one decitabine treatment administrated on day 1 with the starting dose of 30 mg/m².

The methylation was analyzed at three different CpG sites in the MAGE1A promoter by pyrosequencing.

In normal mucosa, the mean methylation value obtained for the three CpG sites was 90% for all patients on day 0 and this value was unchanged in samples taken 11 days later (fig. 4.4.1, upper panel). This trend was also confirmed by the analysis of each CpG site, when the ratio between methylation values at day 11 versus the methylation values of the corresponding site on day 0 were calculated (fig. 4.4.1, lower panel). As is clearly shown, no methylation changes occurred after treatment with decitabine.

In blood samples, MAOEA was methylated to the same extent as the normal mucosa. The methylation of MAOEA in normal mucosa was more extensively affected than normal tissue by treatment with decitabine. However, this is not the case in patients because, in the

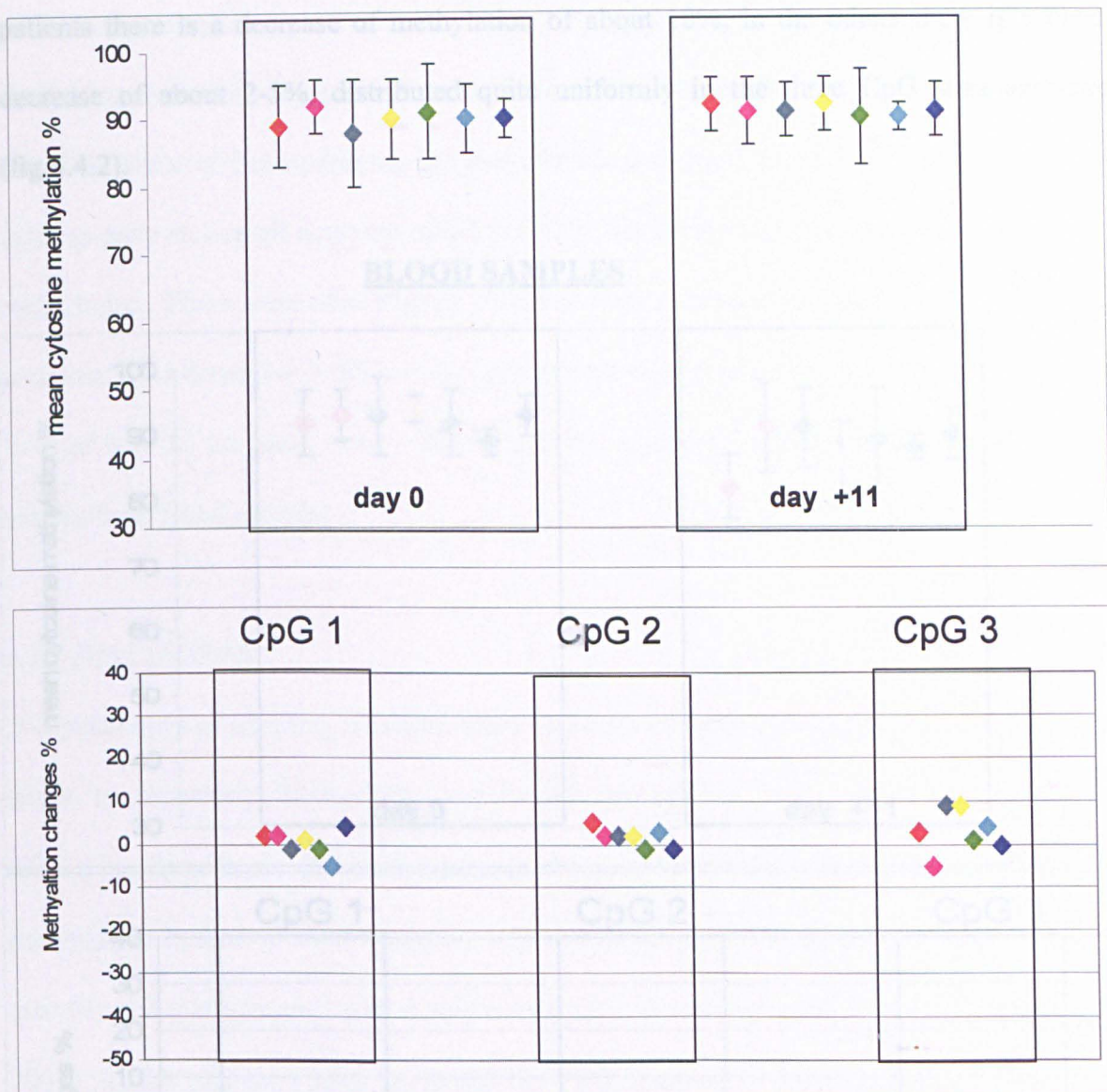


Figure 4.4.1 Methylation analysis of normal biopsies by PSQ

The upper panel shows the average methylation percentage of the cytosines of the three different CpG sites analyzed at the time of diagnosis and 11 days after one treatment with decitabine; the lower panel shows the methylation changes at each CpG site, calculated as ratio of the % methylation on day 11 versus the % methylation of the same site at the time of diagnosis.

A total of seven patients were analyzed and each ascribed a colour.

Figure 4.4.2 Methylation analysis of blood samples by PSQ

See previous figure for details.

In blood samples, MAGE1A was methylated to the same extent as the normal mucosa, but methylation was more extensively affected than normal tissue by treatment with decitabine. However, this is not a general trend in each patient, because, while in two patients there is a decrease of methylation of about 10%, in the others there is a mean decrease of about 2-5%, distributed quite uniformly in the three CpG sites analyzed (fig.4.4.2).

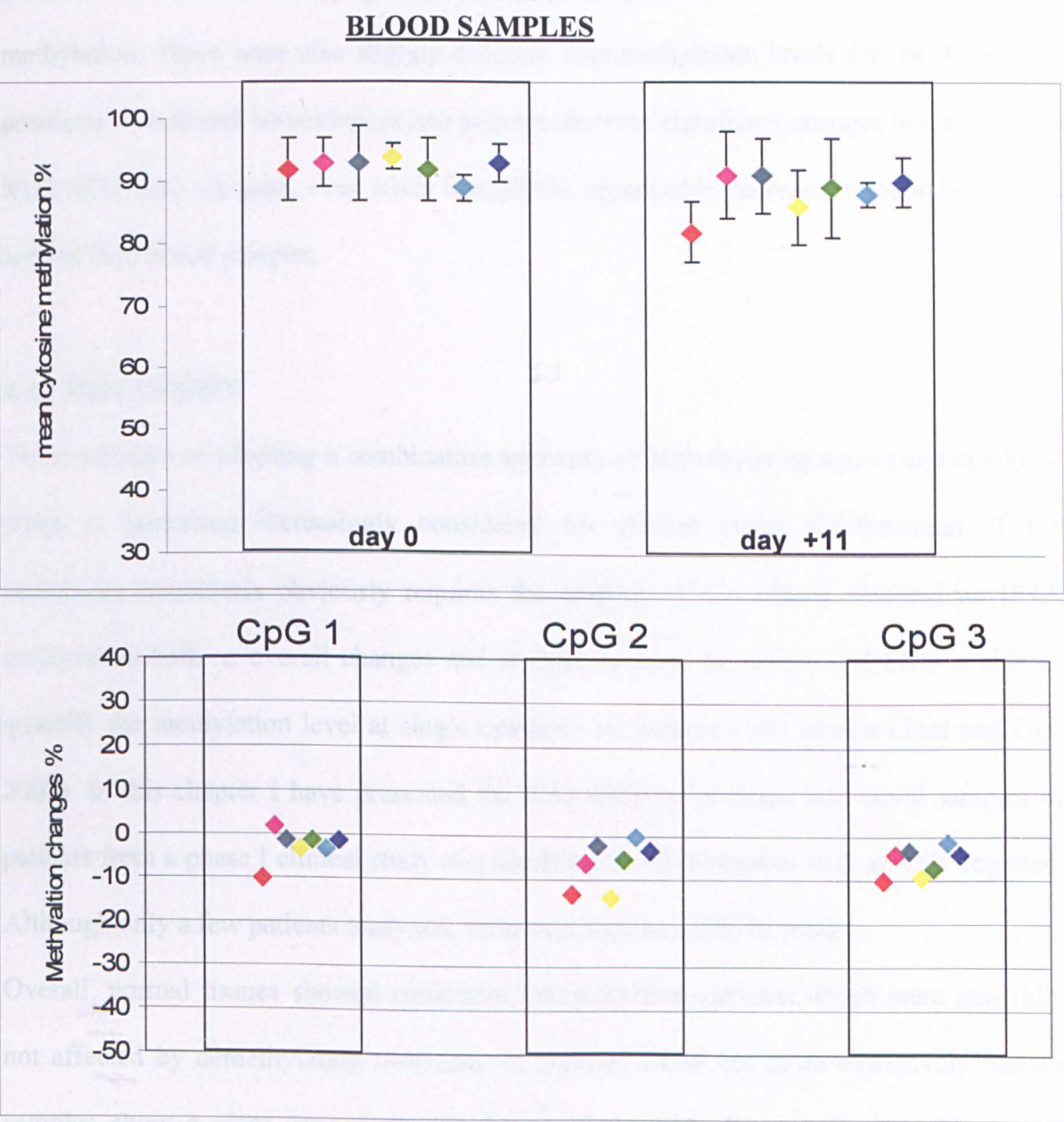


Figure 4.4.2 Methylation analysis of blood samples by PSQ

See previous figure for details.

In tumour biopsies (fig.4.4.3), the analysis showed a very different situation: the level of MAGE 1A methylation was very variable in the untreated samples and this is in general associated to tumour transformation; in particular, except for three patients, the mean methylation level is equal or lower than 80% (fig.4.4.3 upper panel). The effects of decitabine treatment are generally more extensive than in the normal tissue and blood samples. Thus in three patients, the methylation decreased to $\approx 50\%$ or less, while two patients have an overall decrease of 2-5% and in two patients there was a slight increase in methylation. There were also slightly different hypomethylation levels for the three CpG positions considered. Nevertheless two patients show no significant changes in methylation level of MAGE 1A gene, even when a small but appreciable decrease in methylation was seen in their blood samples.

4.4.3 DISCUSSION

The possibility of adopting a combination approach of demethylating agents and cytotoxic drugs is becoming increasingly considered for clinical trials. Confirmation of the underlying hypothesis obviously requires the analysis of the effects obtained on DNA methylation both as overall changes and at selected gene promoters and PSQ is able to quantify the methylation level at single cytosines in selected CpG islands (Tost and Gut, 2007). In this chapter I have presented the PSQ analyses of tissue and blood samples of patients from a phase I clinical study of a combination of decitabine with an ECF regimen. Although only a few patients analyzed, some conclusions could be made.

Overall, normal tissues showed consistent interindividual patterns which were generally not affected by demethylating treatment. In contrast blood but more extensively tumour samples show a clear general demethylation of the MAGE1A CpG sites. This could probably be related to a higher level of proliferation of blood and cancer cells compared to normal cells: decitabine exerts its demethylating function by incorporation into DNA of dividing cells and only in this way prevents the action of DNMTs. In contrast to what was

published in another phase I study (Appleton et al., 2007), I could see a much more extensive but extremely variable demethylation in tumour samples than in blood samples. In this context it is also important to consider the different kind of tumours recruited in the two different studies, which may explain the opposing results. In addition, a lesser effect on blood could also suggest a reduced haematological toxicity and this is would be an advantage rather than a disadvantage.

TUMOUR SAMPLES

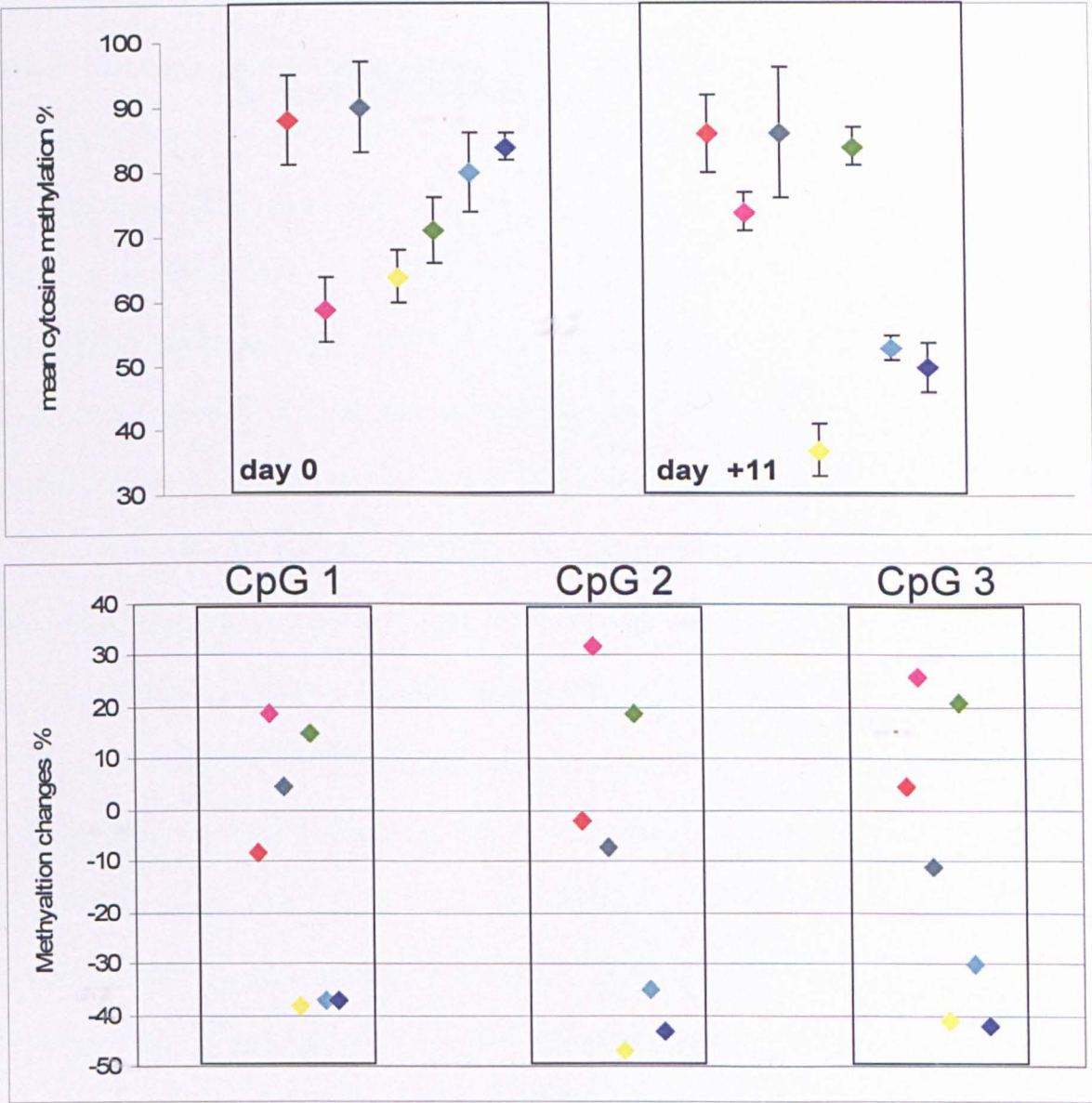


Figure 4.4.3 Methylation analysis of tumour samples by PSQ

See figure 4.4.1 for details.

It is important to emphasise that there are patients whose tumours are clearly unresponsive to decitabine treatment. The reasons for this could be different in different patients and we have to consider that there may be a number of parameters influencing the amount of the effective molecules being incorporated into DNA. Nevertheless the responses in terms of modulating the levels of methylation (5/7) confirmed that one treatment with decitabine is able to exert its effects on MAGE1A promoter methylation in tumour cells.

5. GENERAL DISCUSSION

In the last few years there has been a sustained effort aimed at exploiting the increasing knowledge of the molecular mechanisms leading to tumour formation in order to adopt new therapeutic strategies that will be more specific for cancer cells.

One of the most investigated fields of research has been the epigenomics of cancer, which in essence consists of several reversible chemical modifications affecting the chromatin components, DNA and histone proteins. Thus, molecules that can revert DNA methylation and histone deacetylation have been tested at both the preclinical and clinical level (Yoo and Jones, 2006). At the moment, the efficacy of the oldest DNA demethylating agent, 5-aza-dC cannot be questioned. However characteristics, such as its chemical instability, toxicity and inactivation by cytidine deaminase have prompted the search for and development of other molecules that are able to revert DNA methylation.

In the scientific literature, what has emerged, from testing both nucleoside and non-nucleoside analog DNA methylation inhibitors, is that their efficacy can depend on the cell line being investigated and the methylated promoter being considered (Chuang et al., 2005; Stresemann et al., 2006).

I have shown that the choice of the right demethylating drug is a fundamental step in the preclinical evaluation of the feasibility of combining a DNMT inhibitor with brostallicin, not only for its capacity to induce brostallicin cytotoxicity in the cellular model chosen, but also for the inherent toxicity of the demethylating drug. In general, the use of non-nucleoside analog demethylating molecules did not improve brostallicin activity, although no inherent toxicity was recorded at the concentrations and schedule used. However, because no methylation analysis was performed in this study, it is not possible to evaluate the capability of these molecules to demethylate the GSTP1 promoter or that of other genes. The data available in other studies are controversial. Thus, GSTP1 methylation in LNCaP cells was reverted by a 1-2 week treatment with procainamide *in vitro* and by a weekly treatment for 7 weeks *in vivo* (Lin et al., 2001a). It is therefore possible that a 96h exposure of LNCaP cells to procainamide is not sufficient to cause demethylation nor, in

general, some other general effects that might improve brostallicin cytotoxicity. On the other hand, another study did not confirm the demethylating activity of procainamide on other genes, such as p16 and MAGE1A, and on LINE elements (Chuang et al., 2005).

Zebularine was already known as a cytidine deaminase inhibitor, but its demethylating properties have been investigated only recently (Cheng et al., 2003). Studies showing its application for reverting DNA methylation, especially on solid tumours, have been very few until now and further data are necessary to understand its effects on different genes in different cancer cell types. The demethylating properties of zebularine were firstly demonstrated on the p16 gene in the T24 bladder cancer cell line (both *in vitro* and *in vivo*), but not on the same gene in the prostatic cancer cell line PC3 and in the CALU-1 lung carcinoma cells. However, zebularine was able to exert its demethylating activity at a single-copy gene level (including hMLH1 and RASSF1A genes) and at repeated element level in A2780/ CP70 cells (a cisplatin-resistant ovarian cancer cell line) (Balch et al., 2005; Cheng et al., 2004b; Stresemann et al., 2006).

In contrast, data on methylation inhibition by 5-aza-dC are stronger and more homogeneous and this cytidine analog is usually used as reference molecule in studies characterizing new demethylating agents.

It is important to underline that my project has not been a comparative study of the demethylating properties of different molecules, but rather the investigation of a new kind of therapeutic strategy against prostate cancer, for which few cytotoxic treatments are available. In this context, my study was mainly focused on the *in vivo* effect of the combination of zebularine with brostallicin, in order to establish the possibility of obtaining significant antitumour activity with null or acceptable toxicity. When zebularine and brostallicin were combined, toxicity was not negligible, even though the observed toxicity was rapidly reversed after stopping treatment. The schedule adopted was of prolonged treatments with zebularine in order to generate enhanced brostallicin toxicity in cells re-expressing GSTP1 by constantly maintaining the demethylation induced in

transplanted LNCaP cells. Further studies are necessary to verify whether alternative treatment schedules or doses would have less toxicity while retaining the same efficacy.

The effects of demethylating agents are not gene-specific, so the causes of the increased antitumor activity of the zebularine-brostallicin combination needed to be investigated, starting with the analysis of the expression of the GSTpi protein, which was considered to determine the intracellular activation of brostallicin. Although 5-aza-dC and non nucleoside analog demethylating molecules were tested in the past with the aim of reverting GSTP1 methylation (Lin et al., 2001a), no data were available until now about the effect of zebularine on this gene. The fact that no GSTpi protein was detected in the analyzed samples indicates that zebularine did not exert any demethylating activity on the GSTP1 promoter (in LNCaP cells), thus leaving it in a closed and not-transcriptionally active form, even after prolonged treatments. This “negative” result was confirmed by both the qualitative and quantitative analyses performed.

Actually, if I consider the peculiar mechanism of action of brostallicin, which is inactive as cytotoxic drug if not bound to GSH, it is not straightforward to sustain a general and not well identified role of zebularine in LNCaP sensitization to brostallicin.

Brostallicin is cytotoxic in MCF-7 cells overexpressing GSTmu enzyme (data from Nerviano Medical Sciences, Milan, Italy. Personal communication) and GSTmu is promoter methylated in LNCaP cells (Lodygin et al., 2005). These observations indicate the possible involvement of GSTmu rather than GSTpi in the antitumour activity of brostallicin in LNCaP tumours, after prolonged treatments with zebularine.

Furthermore, it is also important to consider that a demethylating agent does not completely revert the methylation status of a promoter and, usually, the amount of the protein formed is not comparable to that synthesized when a promoter is completely unmethylated. The strong affinity of GSTmu enzyme for brostallicin versus GSTpi could ensure the catalysis of the reaction between GSH and brostallicin even when few GSTmu molecules are synthesized in the cell; in these particular conditions, the GSTmu catalysis

will be undoubtedly more favoured than the same reaction catalyzed by GSTpi or GSTalpha (which is the least efficient among the GST enzymes in the brostallicin activation).

That GSTmu has its promoter methylation partially reverted after *in vivo* treatment with zebularine has been an unexpected finding of this project. For this to be relevant, future experiments will need to demonstrate that this demethylation results in GSTM1 transcription and protein synthesis. At the moment, I have demonstrated that zebularine exerts its demethylating function on GSTM1 rather than on GSTP1 in an *in vivo* system, where factors such as the pharmacokinetics of the drug and the metabolism can affect the proper release of the demethylating drug to tumour cells. Differential demethylating activity may depend on the lower density of methylated cytosines in the GSTM1 than the GSTP1 promoter, thus making it relatively “easier” to revert methylation in GSTM1.

In prostate cancer, only recently, has the analysis of GST expression been widened to other classes of cytosolic GST, such as GSTα and GSTμ (Bostwick et al., 2007). In human prostate cancer, GSTM1 methylation, in a significant percentage of the patients analyzed in a clinical study (Lodygin et al., 2005) and the low levels of expression, makes the results, I obtained in LNCaP cells, more interesting for future combination studies of brostallicin.

This means that tumour transformation of the prostatic tissue is affected by the absence of those protecting systems preventing the DNA to be damaged by exogenous factors and strengthens the role of methylation as a mechanism of gene silencing in the first steps of tumours transformation. The precocity of this event may represent an advantage from the therapeutic point of view, because it could allow hitting the transformed cells when they are still confined to the prostate (Meiers et al., 2007).

From a clinical perspective, zebularine offers the great advantage of being orally administrable (Cheng et al., 2003), and this is a very important characteristic when prolonged treatments are needed.

The combinations of demethylating and cytotoxic drugs have recently become increasingly considered in cancer therapy and the data shown in the last chapter of the results clearly demonstrated the increasing potential for this approach not only in preclinical experimentation, but also in the clinical setting, both on haematological malignancies and on solid tumours (Appleton et al., 2007; Issa, 2007; Plimack et al., 2007).

To reiterate, this approach offers the potential advantage of reverting silencing of methylated promoters, which itself may be therapeutic, and killing cells by specifically expressing proteins that are required for the cytotoxic effects of certain drugs. In this way, the effects of methylation modulators and HDACi can be exploited and a high level of specificity can be achieved. Of course, it will be very important to determine when the lowest levels of methylation are reached, to properly design both preclinical and clinical schedules for combination treatments (Lyko and Brown, 2005) and hence maximize the effects of the administration of a cytotoxic drug. In this regard, my study represents an initial investigation aimed at verifying the feasibility of the combination of zebularine with brostallicin, based on which the schedule of treatment can now be optimized by assensing whether the same demethylating effects can be obtained by lowering zebularine concentration or by using fewer treatments, mainly to reduce the toxicity I have obtained in the schedule of treatment adopted. It is also important to underline that various factors, including DNA hypomethylation and cytotoxicity, may contribute to the complex effects observed after epigenetic drug treatment, and these cannot be neglected in the general evaluation of the effects of a drug combination.

In conclusion my PhD project has demonstrated the feasibility of zebularine combination with a cytotoxic drug. That this combination approach enhances therapy in the model selected indicates that it might also be applied to other kind of tumours, such as the breast cancer cell line MCF-7, which shows the same GSTP1 methylation silencing (Jhaveri and Morrow, 1998).

As knowledge increases on the epigenetic events in tumourigenesis, therapies aimed towards exploiting peculiar modifications need to be properly studied and further improved.

6. REFERENCES

Reference List

- Agah,A., Aghajan,M., Mashayekhi,F., Amini,S., Davis,R.W., Plummer,J.D., Ronaghi,M., and Griffin,P.B. (2004). A multi-enzyme model for Pyrosequencing. *Nucleic Acids Res.* 32, e166.
- Ahmadian,A., Ehn,M., and Hober,S. (2006). Pyrosequencing: history, biochemistry and future. *Clin. Chim. Acta* 363, 83-94.
- Appleton,K., Mackay,H.J., Judson,I., Plumb,J.A., McCormick,C., Strathdee,G., Lee,C., Barrett,S., Reade,S., Jadayel,D., Tang,A., Bellenger,K., Mackay,L., Setanoians,A., Schatzlein,A., Twelves,C., Kaye,S.B., and Brown,R. (2007). Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J. Clin. Oncol.* 25, 4603-4609.
- Armstrong,R.N. (1997). Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* 10, 2-18.
- Bachman,K.E., Park,B.H., Rhee,I., Rajagopalan,H., Herman,J.G., Baylin,S.B., Kinzler,K.W., and Vogelstein,B. (2003). Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell* 3, 89-95.
- Balch,C., Yan,P., Craft,T., Young,S., Skalnik,D.G., Huang,T.H., and Nephew,K.P. (2005). Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. *Mol. Cancer Ther.* 4, 1505-1514.
- Baylin,S.B. and Ohm,J.E. (2006). Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat. Rev. Cancer* 6, 107-116.
- Ben Kasus,T., Ben Zvi,Z., Marquez,V.E., Kelley,J.A., and Agbaria,R. (2005). Metabolic activation of zebularine, a novel DNA methylation inhibitor, in human bladder carcinoma cells. *Biochem. Pharmacol.* 70, 121-133.
- Berger,S.L. (2007). The complex language of chromatin regulation during transcription. *Nature* 447, 407-412.
- Bernstein,B.E., Meissner,A., and Lander,E.S. (2007). The mammalian epigenome. *Cell* 128, 669-681.
- Bird,A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* 16, 6-21.
- Bjornsson,H.T., Fallin,M.D., and Feinberg,A.P. (2004). An integrated epigenetic and genetic approach to common human disease. *Trends Genet.* 20, 350-358.
- Bostwick,D.G., Meiers,I., and Shanks,J.H. (2007). Glutathione S-transferase: differential expression of alpha, mu, and pi isoenzymes in benign prostate, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma. *Hum. Pathol.* 38, 1394-1401.
- Broggini,M., Marchini,S., Fontana,E., Moneta,D., Fowst,C., and Geroni,C. (2004). Brostallicin: a new concept in minor groove DNA binder development. *Anticancer Drugs* 15, 1-6.
- Brueckner,B., Boy,R.G., Siedlecki,P., Musch,T., Kliem,H.C., Zielenkiewicz,P., Suhai,S., Wiessler,M., and Lyko,F. (2005). Epigenetic reactivation of tumor suppressor genes by a

novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res.* 65, 6305-6311.

Cameron,E.E., Bachman,K.E., Myohanen,S., Herman,J.G., and Baylin,S.B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* 21, 103-107.

Cheng,J.C., Matsen,C.B., Gonzales,F.A., Ye,W., Greer,S., Marquez,V.E., Jones,P.A., and Selker,E.U. (2003). Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J. Natl. Cancer Inst.* 95, 399-409.

Cheng,J.C., Weisenberger,D.J., Gonzales,F.A., Liang,G., Xu,G.L., Hu,Y.G., Marquez,V.E., and Jones,P.A. (2004a). Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. *Mol. Cell Biol.* 24, 1270-1278.

Cheng,J.C., Yoo,C.B., Weisenberger,D.J., Chuang,J., Wozniak,C., Liang,G., Marquez,V.E., Greer,S., Orntoft,T.F., Thykjaer,T., and Jones,P.A. (2004b). Preferential response of cancer cells to zebularine. *Cancer Cell* 6, 151-158.

Cho,S.G., Lee,Y.H., Park,H.S., Ryoo,K., Kang,K.W., Park,J., Eom,S.J., Kim,M.J., Chang,T.S., Choi,S.Y., Shim,J., Kim,Y., Dong,M.S., Lee,M.J., Kim,S.G., Ichijo,H., and Choi,E.J. (2001). Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. *J. Biol. Chem.* 276, 12749-12755.

Christman,J.K. (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21, 5483-5495.

Chuang,J.C., Yoo,C.B., Kwan,J.M., Li,T.W., Liang,G., Yang,A.S., and Jones,P.A. (2005). Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Mol. Cancer Ther.* 4, 1515-1520.

Clark,S.J., Harrison,J., Paul,C.L., and Frommer,M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* 22, 2990-2997.

Clark,S.J. and Melki,J. (2002). DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene* 21, 5380-5387.

D'Incalci,M. and Sessa,C. (1997). DNA minor groove binding ligands: a new class of anticancer agents. *Expert. Opin. Investig. Drugs* 6, 875-884.

Dancey,J.E. and Chen,H.X. (2006). Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat. Rev. Drug Discov.* 5, 649-659.

Dang,D.T., Chen,F., Kohli,M., Rago,C., Cummins,J.M., and Dang,L.H. (2005). Glutathione S-transferase pi 1 promotes tumorigenicity in HCT116 human colon cancer cells. *Cancer Res.* 65, 9485-9494.

De Marzo,A.M., DeWeese,T.L., Platz,E.A., Meeker,A.K., Nakayama,M., Epstein,J.I., Isaacs,W.B., and Nelson,W.G. (2004). Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. *J. Cell Biochem.* 91, 459-477.

DeMarzo,A.M., Nelson,W.G., Isaacs,W.B., and Epstein,J.I. (2003). Pathological and molecular aspects of prostate cancer. *Lancet* 361, 955-964.

- Denizot,F. and Lang,R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89, 271-277.
- Dobosy,J.R., Roberts,J.L., Fu,V.X., and Jarrard,D.F. (2007). The expanding role of epigenetics in the development, diagnosis and treatment of prostate cancer and benign prostatic hyperplasia. *J. Urol.* 177, 822-831.
- Dong,A., Yoder,J.A., Zhang,X., Zhou,L., Bestor,T.H., and Cheng,X. (2001). Structure of human DNMT2, an enigmatic DNA methyltransferase homolog that displays denaturant-resistant binding to DNA. *Nucleic Acids Res.* 29, 439-448.
- Driscoll,J.S., Marquez,V.E., Plowman,J., Liu,P.S., Kelley,J.A., and Barchi,J.J., Jr. (1991). Antitumor properties of 2(1H)-pyrimidinone riboside (zebularine) and its fluorinated analogues. *J. Med. Chem.* 34, 3280-3284.
- Espada,J., Ballestar,E., Fraga,M.F., Villar-Garea,A., Juarranz,A., Stockert,J.C., Robertson,K.D., Fuks,F., and Esteller,M. (2004). Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. *J. Biol. Chem.* 279, 37175-37184.
- Esteller,M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 21, 5427-5440.
- Esteller,M. (2007). Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* 8, 286-298.
- Esteller,M., Corn,P.G., Urena,J.M., Gabrielson,E., Baylin,S.B., and Herman,J.G. (1998). Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res.* 58, 4515-4518.
- Fahrner,J.A., Eguchi,S., Herman,J.G., and Baylin,S.B. (2002). Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. *Cancer Res.* 62, 7213-7218.
- Fang,M.Z., Wang,Y., Ai,N., Hou,Z., Sun,Y., Lu,H., Welsh,W., and Yang,C.S. (2003). Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* 63, 7563-7570.
- Fedier,A., Fowst,C., Tursi,J., Geroni,C., Haller,U., Marchini,S., and Fink,D. (2003). Brostallicin (PNU-166196)--a new DNA minor groove binder that retains sensitivity in DNA mismatch repair-deficient tumour cells. *Br. J. Cancer* 89, 1559-1565.
- Feinberg,A.P. (2007). Phenotypic plasticity and the epigenetics of human disease. *Nature* 447, 433-440.
- Feinberg,A.P., Ohlsson,R., and Henikoff,S. (2006). The epigenetic progenitor origin of human cancer. *Nat. Rev. Genet.* 7, 21-33.
- Feinberg,A.P. and Tycko,B. (2004). The history of cancer epigenetics. *Nat. Rev. Cancer* 4, 143-153.
- Feldman,B.J. and Feldman,D. (2001). The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* 1, 34-45.

- Frigola,J., Song,J., Stirzaker,C., Hinshelwood,R.A., Peinado,M.A., and Clark,S.J. (2006). Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. *Nat. Genet.* 38, 540-549.
- Gardiner-Garden,M. and Frommer,M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* 196, 261-282.
- Gate,L., Majumdar,R.S., Lunk,A., and Tew,K.D. (2004). Increased myeloproliferation in glutathione S-transferase pi-deficient mice is associated with a deregulation of JNK and Janus kinase/STAT pathways. *J. Biol. Chem.* 279, 8608-8616.
- Gate,L., Majumdar,R.S., Lunk,A., and Tew,K.D. (2005). Influence of glutathione S-transferase pi and p53 expression on tumor frequency and spectrum in mice. *Int. J. Cancer* 113, 29-35.
- Gaudet,F., Hodgson,J.G., Eden,A., Jackson-Grusby,L., Dausman,J., Gray,J.W., Leonhardt,H., and Jaenisch,R. (2003). Induction of tumors in mice by genomic hypomethylation. *Science* 300, 489-492.
- Geroni,C., Marchini,S., Cozzi,P., Galliera,E., Ragg,E., Colombo,T., Battaglia,R., Howard,M., D'Incalci,M., and Broggin,M. (2002). Brostallicin, a novel anticancer agent whose activity is enhanced upon binding to glutathione. *Cancer Res.* 62, 2332-2336.
- Goffin,J. and Eisenhauer,E. (2002). DNA methyltransferase inhibitors-state of the art. *Ann. Oncol.* 13, 1699-1716.
- Goll,M.G. and Bestor,T.H. (2005). Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* 74, 481-514.
- Goll,M.G., Kirpekar,F., Maggert,K.A., Yoder,J.A., Hsieh,C.L., Zhang,X., Golic,K.G., Jacobsen,S.E., and Bestor,T.H. (2006). Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science* 311, 395-398.
- Gonzalgo,M.L., Pavlovich,C.P., Lee,S.M., and Nelson,W.G. (2003). Prostate cancer detection by GSTP1 methylation analysis of postbiopsy urine specimens. *Clin. Cancer Res.* 9, 2673-2677.
- Grewal,S.I. and Moazed,D. (2003). Heterochromatin and epigenetic control of gene expression. *Science* 301, 798-802.
- Habig,W.H. and Jakoby,W.B. (1981). Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* 77, 398-405.
- Hadaschik,B.A. and Gleave,M.E. (2007). Therapeutic options for hormone-refractory prostate cancer in 2007. *Urol. Oncol.* 25, 413-419.
- Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.
- Harden,S.V., Guo,Z., Epstein,J.I., and Sidransky,D. (2003). Quantitative GSTP1 methylation clearly distinguishes benign prostatic tissue and limited prostate adenocarcinoma. *J. Urol.* 169, 1138-1142.
- Hatchwell,E. and Greally,J.M. (2007). The potential role of epigenomic dysregulation in complex human disease. *Trends Genet.* 23, 588-595.

- Hayes, J.D., Flanagan, J.U., and Jowsey, I.R. (2005). Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **45**, 51-88.
- Henderson, C.J. and Wolf, C.R. (2005). Disruption of the glutathione transferase pi class genes. *Methods Enzymol.* **401**, 116-135.
- Henrique, R. and Jeronimo, C. (2004). Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. *Eur. Urol.* **46**, 660-669.
- Herman, J.G. and Baylin, S.B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042-2054.
- Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., and Baylin, S.B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. U. S. A* **93**, 9821-9826.
- Hopkins, T.G., Burns, P.A., and Routledge, M.N. (2007). DNA methylation of GSTP1 as biomarker in diagnosis of prostate cancer. *Urology* **69**, 11-16.
- Horwich, A. (2006). Systemic treatment for prostate cancer. *Ann. Oncol.* **17 Suppl 10**, x211-x213.
- Issa, J.P. (2004). CpG island methylator phenotype in cancer. *Nat. Rev. Cancer* **4**, 988-993.
- Issa, J.P. (2007). DNA methylation as a therapeutic target in cancer. *Clin. Cancer Res.* **13**, 1634-1637.
- Issa, J.P., Garcia-Manero, G., Giles, F.J., Mannari, R., Thomas, D., Faderl, S., Bayar, E., Lyons, J., Rosenfeld, C.S., Cortes, J., and Kantarjian, H.M. (2004). Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* **103**, 1635-1640.
- Jackson-Grusby, L., Laird, P.W., Magge, S.N., Moeller, B.J., and Jaenisch, R. (1997). Mutagenicity of 5-aza-2'-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl. Acad. Sci. U. S. A* **94**, 4681-4685.
- Jaenisch, R. and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33 Suppl**, 245-254.
- Jhaveri, M.S. and Morrow, C.S. (1998). Methylation-mediated regulation of the glutathione S-transferase P1 gene in human breast cancer cells. *Gene* **210**, 1-7.
- Johnstone, R.W. (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat. Rev. Drug Discov.* **1**, 287-299.
- Jones, P.A. and Baylin, S.B. (2002). The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3**, 415-428.
- Jones, P.A. and Baylin, S.B. (2007). The epigenomics of cancer. *Cell* **128**, 683-692.
- Jones, P.A. and Taylor, S.M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**, 85-93.
- Juttermann, R., Li, E., and Jaenisch, R. (1994). Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. U. S. A* **91**, 11797-11801.

- Karan,D., Lin,M.F., Johansson,S.L., and Batra,S.K. (2003). Current status of the molecular genetics of human prostatic adenocarcinomas. *Int. J. Cancer* 103, 285-293.
- Karpf,A.R. and Jones,D.A. (2002). Reactivating the expression of methylation silenced genes in human cancer. *Oncogene* 21, 5496-5503.
- Kato,T., Yamano,Y., Tsuji,M., and Watanabe,M. (2008). Genetic polymorphisms of human cytosol glutathione S-transferases and prostate cancer. *Pharmacogenomics*. 9, 93-104.
- Khorasanizadeh,S. (2004). The nucleosome: from genomic organization to genomic regulation. *Cell* 116, 259-272.
- Kinzler,K.W. and Vogelstein,B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87, 159-170.
- Klose,R.J. and Bird,A.P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* 31, 89-97.
- Knudson,A.G. (2000). Chasing the cancer demon. *Annu. Rev. Genet.* 34, 1-19.
- Kouzarides,T. (2007). Chromatin modifications and their function. *Cell* 128, 693-705.
- Laird,P.W. (2003). The power and the promise of DNA methylation markers. *Nat. Rev. Cancer* 3, 253-266.
- Lande-Diner,L. and Cedar,H. (2005). Silence of the genes--mechanisms of long-term repression. *Nat. Rev. Genet.* 6, 648-654.
- Leahy,M., Ray-Coquard,I., Verweij,J., Le Cesne,A., Duffaud,F., Hogendoorn,P.C., Fowst,C., de Balincourt,C., di Paola,E.D., van Glabbeke,M., Judson,I., and Blay,J.Y. (2007). Brostallicin, an agent with potential activity in metastatic soft tissue sarcoma: a phase II study from the European Organisation for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. *Eur. J. Cancer* 43, 308-315.
- Lee,B.H., Yegnasubramanian,S., Lin,X., and Nelson,W.G. (2005). Procainamide is a specific inhibitor of DNA methyltransferase 1. *J. Biol. Chem.* 280, 40749-40756.
- Lee,W.H., Morton,R.A., Epstein,J.I., Brooks,J.D., Campbell,P.A., Bova,G.S., Hsieh,W.S., Isaacs,W.B., and Nelson,W.G. (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. U. S. A* 91, 11733-11737.
- Lemaire,M., Momparler,L.F., Bernstein,M.L., Marquez,V.E., and Momparler,R.L. (2005). Enhancement of antineoplastic action of 5-aza-2'-deoxycytidine by zebularine on L1210 leukemia. *Anticancer Drugs* 16, 301-308.
- Leu,Y.W., Rahmatpanah,F., Shi,H., Wei,S.H., Liu,J.C., Yan,P.S., and Huang,T.H. (2003). Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. *Cancer Res.* 63, 6110-6115.
- Li,E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.* 3, 662-673.

- Li, L.C., Carroll, P.R., and Dahiya, R. (2005). Epigenetic changes in prostate cancer: implication for diagnosis and treatment. *J. Natl. Cancer Inst.* 97, 103-115.
- Li, L.C. and Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics*. 18, 1427-1431.
- Li, L.C., Okino, S.T., and Dahiya, R. (2004). DNA methylation in prostate cancer. *Biochim. Biophys. Acta* 1704, 87-102.
- Lin, X., Asgari, K., Putzi, M.J., Gage, W.R., Yu, X., Cornblatt, B.S., Kumar, A., Piantadosi, S., DeWeese, T.L., De Marzo, A.M., and Nelson, W.G. (2001a). Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* 61, 8611-8616.
- Lin, X., Tascilar, M., Lee, W.H., Vles, W.J., Lee, B.H., Veeraswamy, R., Asgari, K., Freije, D., van Rees, B., Gage, W.R., Bova, G.S., Isaacs, W.B., Brooks, J.D., DeWeese, T.L., De Marzo, A.M., and Nelson, W.G. (2001b). GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells. *Am. J. Pathol.* 159, 1815-1826.
- Lodygin, D., Epanchintsev, A., Menssen, A., Diebold, J., and Hermeking, H. (2005). Functional epigenomics identifies genes frequently silenced in prostate cancer. *Cancer Res.* 65, 4218-4227.
- Lund, A.H. and van Lohuizen, M. (2004). Epigenetics and cancer. *Genes Dev.* 18, 2315-2335.
- Lyko, F. and Brown, R. (2005). DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. *J. Natl. Cancer Inst.* 97, 1498-1506.
- Machover, D., Zittoun, J., Saffroy, R., Broet, P., Giraudier, S., Magnaldo, T., Goldschmidt, E., Debuire, B., Orrico, M., Tan, Y., Mishal, Z., Chevallier, O., Tonetti, C., Jouault, H., Ulusakarya, A., Tanguy, M.L., Metzger, G., and Hoffman, R.M. (2002). Treatment of cancer cells with methioninase produces DNA hypomethylation and increases DNA synthesis. *Cancer Res.* 62, 4685-4689.
- Marquez, V.E., Barchi, J.J., Jr., Kelley, J.A., Rao, K.V., Agbaria, R., Ben Kasus, T., Cheng, J.C., Yoo, C.B., and Jones, P.A. (2005). Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. The magic of its chemistry and biology. *Nucleosides Nucleotides Nucleic Acids* 24, 305-318.
- McIlwain, C.C., Townsend, D.M., and Tew, K.D. (2006). Glutathione S-transferase polymorphisms: cancer incidence and therapy. *Oncogene* 25, 1639-1648.
- Meiers, I., Shanks, J.H., and Bostwick, D.G. (2007). Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. *Pathology* 39, 299-304.
- Millar, D.S., Ow, K.K., Paul, C.L., Russell, P.J., Molloy, P.L., and Clark, S.J. (1999). Detailed methylation analysis of the glutathione S-transferase pi (GSTP1) gene in prostate cancer. *Oncogene* 18, 1313-1324.
- Millar, D.S., Paul, C.L., Molloy, P.L., and Clark, S.J. (2000). A distinct sequence (ATAAA)_n separates methylated and unmethylated domains at the 5'-end of the GSTP1 CpG island. *J. Biol. Chem.* 275, 24893-24899.

- Minucci,S. and Pelicci,P.G. (2006). Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* 6, 38-51.
- Momparler,R.L. (1985). Molecular, cellular and animal pharmacology of 5-aza-2'-deoxycytidine. *Pharmacol. Ther.* 30, 287-299.
- Momparler,R.L. and Bovenzi,V. (2000). DNA methylation and cancer. *J. Cell Physiol* 183, 145-154.
- Moscow,J.A., Townsend,A.J., and Cowan,K.H. (1989). Elevation of pi class glutathione S-transferase activity in human breast cancer cells by transfection of the GST pi gene and its effect on sensitivity to toxins. *Mol. Pharmacol.* 36, 22-28.
- Mosmann,T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55-63.
- Nakayama,M., Bennett,C.J., Hicks,J.L., Epstein,J.I., Platz,E.A., Nelson,W.G., and De Marzo,A.M. (2003). Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am. J. Pathol.* 163, 923-933.
- Nelson,W.G., De Marzo,A.M., and Isaacs,W.B. (2003). Prostate cancer. *N. Engl. J. Med.* 349, 366-381.
- Nieto,M., Samper,E., Fraga,M.F., Gonzalez,d.B., Esteller,M., and Serrano,M. (2004). The absence of p53 is critical for the induction of apoptosis by 5-aza-2'-deoxycytidine. *Oncogene* 23, 735-743.
- Ohman,F.K. and Nordqvist,K. (2001). The melanoma antigen genes--any clues to their functions in normal tissues? *Exp. Cell Res.* 265, 185-194.
- Palapattu,G.S., Sutcliffe,S., Bastian,P.J., Platz,E.A., De Marzo,A.M., Isaacs,W.B., and Nelson,W.G. (2005). Prostate carcinogenesis and inflammation: emerging insights. *Carcinogenesis* 26, 1170-1181.
- Perry,A.S., Foley,R., Woodson,K., and Lawler,M. (2006). The emerging roles of DNA methylation in the clinical management of prostate cancer. *Endocr. Relat Cancer* 13, 357-377.
- Plimack,E.R., Stewart,D.J., and Issa,J.P. (2007). Combining epigenetic and cytotoxic therapy in the treatment of solid tumors. *J. Clin. Oncol.* 25, 4519-4521.
- Plumb,J.A., Strathdee,G., Sludden,J., Kaye,S.B., and Brown,R. (2000). Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res.* 60, 6039-6044.
- Rebbeck,T.R., Walker,A.H., Jaffe,J.M., White,D.L., Wein,A.J., and Malkowicz,S.B. (1999). Glutathione S-transferase-mu (GSTM1) and -theta (GSTT1) genotypes in the etiology of prostate cancer. *Cancer Epidemiol. Biomarkers Prev.* 8, 283-287.
- Reik,W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 447, 425-432.

- Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Lengauer, C., Kinzler, K.W., Baylin, S.B., and Vogelstein, B. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416, 552-556.
- Richardson, B. (2003). DNA methylation and autoimmune disease. *Clin. Immunol.* 109, 72-79.
- Robert, M.F., Morin, S., Beaulieu, N., Gauthier, F., Chute, I.C., Barsalou, A., and MacLeod, A.R. (2003). DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat. Genet.* 33, 61-65.
- Roberts, C.W. and Orkin, S.H. (2004). The SWI/SNF complex--chromatin and cancer. *Nat. Rev. Cancer* 4, 133-142.
- Robertson, K.D. (2002). DNA methylation and chromatin - unraveling the tangled web. *Oncogene* 21, 5361-5379.
- Robertson, K.D. (2005). DNA methylation and human disease. *Nat. Rev. Genet.* 6, 597-610.
- Rushmore, T.H. and Pickett, C.B. (1993). Glutathione S-transferases, structure, regulation, and therapeutic implications. *J. Biol. Chem.* 268, 11475-11478.
- Sabatino, M.A., Colombo, T., Geroni, C., Marchini, S., and Broggin, M. (2003). Enhancement of in vivo antitumor activity of classical anticancer agents by combination with the new, glutathione-interacting DNA minor groove-binder, brostallicin. *Clin. Cancer Res.* 9, 5402-5408.
- Sasaki, H. and Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat. Rev. Genet.* 9, 129-140.
- Schaefer, C.B., Ooi, S.K., Bestor, T.H., and Bourc'his, D. (2007). Epigenetic decisions in mammalian germ cells. *Science* 316, 398-399.
- Schulz, W.A. and Hatina, J. (2006). Epigenetics of prostate cancer: beyond DNA methylation. *J. Cell Mol. Med.* 10, 100-125.
- Schwartzmann, G., Schunemann, H., Gorini, C.N., Filho, A.F., Garbino, C., Sabini, G., Muse, I., DiLeone, L., and Mans, D.R. (2000). A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest New Drugs* 18, 83-91.
- Segura-Pacheco, B., Trejo-Becerril, C., Perez-Cardenas, E., Taja-Chayeb, L., Mariscal, I., Chavez, A., Acuna, C., Salazar, A.M., Lizano, M., and Duenas-Gonzalez, A. (2003). Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. *Clin. Cancer Res.* 9, 1596-1603.
- Song, J.Z., Stirzaker, C., Harrison, J., Melki, J.R., and Clark, S.J. (2002). Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells. *Oncogene* 21, 1048-1061.

Stirzaker,C., Song,J.Z., Davidson,B., and Clark,S.J. (2004). Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. *Cancer Res.* 64, 3871-3877.

Stresemann,C., Brueckner,B., Musch,T., Stopper,H., and Lyko,F. (2006). Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res.* 66, 2794-2800.

Takai,D. and Jones,P.A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl. Acad. Sci. U. S. A* 99, 3740-3745.

Ten Tije,A.J., Verweij,J., Sparreboom,A., Van Der,G.A., Fowst,C., Fiorentini,F., Tursi,J., Antonellini,A., Mantel,M., Hartman,C.M., Stoter,G., Planting,A.S., and De Jonge,M.J. (2003). Phase I and pharmacokinetic study of brostallicin (PNU-166196), a new DNA minor-groove binder, administered intravenously every 3 weeks to adult patients with metastatic cancer. *Clin. Cancer Res.* 9, 2957-2964.

Teodoridis,J.M., Hall,J., Marsh,S., Kannall,H.D., Smyth,C., Curto,J., Siddiqui,N., Gabra,H., McLeod,H.L., Strathdee,G., and Brown,R. (2005). CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res.* 65, 8961-8967.

Tew,K.D. (1994). Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res.* 54, 4313-4320.

Tew,K.D., Monks,A., Barone,L., Rosser,D., Akerman,G., Montali,J.A., Wheatley,J.B., and Schmidt,D.E., Jr. (1996). Glutathione-associated enzymes in the human cell lines of the National Cancer Institute Drug Screening Program. *Mol. Pharmacol.* 50, 149-159.

Ting,A.H., Jair,K.W., Suzuki,H., Yen,R.W., Baylin,S.B., and Schuebel,K.E. (2004). CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1. *Nat. Genet.* 36, 582-584.

Ting,A.H., McGarvey,K.M., and Baylin,S.B. (2006). The cancer epigenome--components and functional correlates. *Genes Dev.* 20, 3215-3231.

Tokumaru,Y., Harden,S.V., Sun,D.I., Yamashita,K., Epstein,J.I., and Sidransky,D. (2004). Optimal use of a panel of methylation markers with GSTP1 hypermethylation in the diagnosis of prostate adenocarcinoma. *Clin. Cancer Res.* 10, 5518-5522.

Tost,J. and Gut,I.G. (2007). DNA methylation analysis by pyrosequencing. *Nat. Protoc.* 2, 2265-2275.

Townsend,D.M., Findlay,V.L., and Tew,K.D. (2005). Glutathione S-transferases as regulators of kinase pathways and anticancer drug targets. *Methods Enzymol.* 401, 287-307.

Townsend,D.M. and Tew,K.D. (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* 22, 7369-7375.

Townsend,D.M., Tew,K.D., and Tapiero,H. (2003). The importance of glutathione in human disease. *Biomed. Pharmacother.* 57, 145-155.

Vanyushin,B.F. (2005). Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell. *Biochemistry (Mosc.)* 70, 488-499.

- Villar-Garea,A., Fraga,M.F., Espada,J., and Esteller,M. (2003). Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. *Cancer Res.* 63, 4984-4989.
- Wang,L.G., Beklemisheva,A., Liu,X.M., Ferrari,A.C., Feng,J., and Chiao,J.W. (2007). Dual action on promoter demethylation and chromatin by an isothiocyanate restored GSTP1 silenced in prostate cancer. *Mol. Carcinog.* 46, 24-31.
- Wang,T., Arifoglu,P., Ronai,Z., and Tew,K.D. (2001). Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *J. Biol. Chem.* 276, 20999-21003.
- Waxman,D.J. (1990). Glutathione S-transferases: role in alkylating agent resistance and possible target for modulation chemotherapy--a review. *Cancer Res.* 50, 6449-6454.
- Weisenberger,D.J., Velicescu,M., Cheng,J.C., Gonzales,F.A., Liang,G., and Jones,P.A. (2004). Role of the DNA methyltransferase variant DNMT3b3 in DNA methylation. *Mol. Cancer Res.* 2, 62-72.
- Wilson,A.S., Power,B.E., and Molloy,P.L. (2007). DNA hypomethylation and human diseases. *Biochim. Biophys. Acta* 1775, 138-162.
- Yamanaka,M., Watanabe,M., Yamada,Y., Takagi,A., Murata,T., Takahashi,H., Suzuki,H., Ito,H., Tsukino,H., Katoh,T., Sugimura,Y., and Shiraishi,T. (2003). Altered methylation of multiple genes in carcinogenesis of the prostate. *Int. J. Cancer* 106, 382-387.
- Yegnasubramanian,S., Kowalski,J., Gonzalgo,M.L., Zahurak,M., Piantadosi,S., Walsh,P.C., Bova,G.S., De Marzo,A.M., Isaacs,W.B., and Nelson,W.G. (2004). Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res.* 64, 1975-1986.
- Yoo,C.B., Jeong,S., Egger,G., Liang,G., Phiasivongsa,P., Tang,C., Redkar,S., and Jones,P.A. (2007). Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. *Cancer Res.* 67, 6400-6408.
- Yoo,C.B. and Jones,P.A. (2006). Epigenetic therapy of cancer: past, present and future. *Nat. Rev. Drug Discov.* 5, 37-50.
- Zhou,L., Cheng,X., Connolly,B.A., Dickman,M.J., Hurd,P.J., and Hornby,D.P. (2002). Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *J. Mol. Biol.* 321, 591-599.

7. APPENDIX

7.1 LIST OF ABBREVIATIONS

| | |
|----------|--------------------------------------|
| aa | aminoacid |
| AHO | Albright Hereditary Osteodystrophy |
| ANOVA | Analysis of Variance |
| APC | Adenomatosis Polyposis Coli |
| APS | Ammonium Persulphate |
| AR | Androgen Receptor |
| ART | Assisted Reproductive Technologies |
| AS | Angelman Syndrome |
| ASK | Apoptosis Signal Regulating Kinase1 |
| ASO | Antisense Oligonucleotides |
| ATP | adenosine 5' triphosphate |
| 5-azaCR | 5-azacytidine |
| 5-aza-dC | 5-aza-2'-deoxycytidine |
| bp | base pairs |
| BPH | Benign Prostatic Hyperplasia |
| BSA | bovine serum albumin |
| BWS | Beckwith-Wiedemann Syndrome |
| CAGE | Cancer Associated Gene |
| cDNA | complementary deoxyribonucleic acid |
| cDDP | cis-dichloro-diamine-platinum |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| CDNK2A | Cyclin-dependent kinase inhibitor 2A |
| CIMP | CpG island Methylator Phenotype |
| DMBA | Dimethylbenzanthracene |
| DMR | Differentially Methylated Region |
| DMSO | dimethylsulphoxide |

| | |
|------------------|--|
| DNA | deoxyribonucleic acid |
| dNTP | nucleotides |
| DNMT | DNA methyltransferase |
| DST | distamycin |
| DTT | dithiothreitol |
| EA | ethacrynic acid |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| ERK | Extracellular signal Regulating Kinase |
| ET-A | Endothelin - A |
| FCS | fetal calf serum |
| FLAP | 5-lipoxygenase activating protein |
| FRAXA | Fragile X Syndrome |
| GnRH | Gonadotropin releasing Hormone |
| GSH | glutathione |
| GST | Glutathione S-transferase |
| h | hour |
| HAT | Histone acetyl transferase |
| HDAC | Histone deacetylase |
| HGPIN | High Grade Prostatic Intraepithelial Neoplasia |
| HIC | Hypermethylated in Cancer |
| H3K9 | Histone3Lysine9 |
| HRPC | Hormone Refractory Prostate Cancer |
| HSP | Heat Shock Protein |
| IC ₅₀ | concentration inhibiting the growth by 50% |
| ICR | Imprinting control region |
| IGF | Insuline Growth Factor |
| IgG | immunoglobulin |
| IHC | Immunohistochemistry |
| ip | intraperitoneus |
| IVM | In Vitro Methylated |
| JNK | c-Jun-N-terminal Kinase |
| Kb | kilobase pairs |
| KDa | kilodaltons |
| KO | knock-out |
| l | liter |

| | |
|--------|--|
| LB | Luria–Bertani |
| LINE | Long Interspersed Nuclear Element |
| LOI | Loss of Imprinting |
| LRES | Long Range Epigenetic Silencing |
| LTC4S | Leukotriene C4 Syntase |
| MAAI | Maleylacetoacetate Isomerase |
| MAGE1A | Melanoma Antigen Family 1A |
| MAPEG | Membrane Associated Proteins in Eicosanoid and Glutathione Metabolism |
| MAPK | Mitogen Activated Stress Kinase |
| MBP | Methyl Binding Protein |
| MDR | Multi Drug Resistance |
| MGB | Minor Groove Binder |
| MGMT | O6-methylguanine DNA methyltransferase |
| MSP | Methylation Specific PCR |
| mRNA | messenger ribonucleic acid |
| NSCLC | Non Small Cell Lung Cancer |
| o/n | over night |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCa | Prostate Cancer |
| PCA | procaine |
| PCR | polymerase chain reaction |
| PDGF | Platelet-derived Growth Factor receptor |
| PGES | Prostaglandin E2 synthase 1 |
| PHP1A | Pseudohypoparathyroidism |
| PIA | Proliferative Inflammatory Atrophy |
| PLAU | Urokinase Plasminogen Activator |
| PMSF | phenylmethysulphonyl fluoride |
| PSA | Prostate Specific Antigen |
| PSQ | Pyrosequencing |
| PTEN | Phosphate and Tensine homologue |
| PVDF | Polyvinylidene Fluoride |
| PWS | Prader Willy Syndrome |
| RAR | Retinoic Acid Receptor |
| RB | Retinoblastoma |

| | |
|-------|---------------------------------------|
| RNA | ribonucleic acid |
| ROS | Reactive oxygen species |
| sc | sub-cutaneously |
| SD | standard deviation |
| SDS | sodium dodecylsulphate |
| SLE | Systemic Lupus Erythematosus |
| TAM | tallimustine |
| TBS | tris buffered saline |
| TEMED | N',N,N',N'-tetramethylethylenediamine |
| TI | tumor growth inhibition |
| TSA | Trichostatin A |
| TSS | Transcription Start Site |
| UV | ultra violet |
| UTR | untranslated region |
| V | Volts |
| VEGF | Vascular Endothelial Growth Factor |
| w/v | weight/volume |
| wt | wild type |

7.2 LIST OF PUBLICATIONS

Full publications by the candidate on topics not associated with the work described here

M Marabese, S Marchini, **MA Sabatino**, F Polato, F Vikhanskaya, E Marrazzo, E Riccardi, E Scanziani and M Broggin (2005)

Effects of inducible overexpression of DNp73 alpha on cancer cell growth and response to treatment *in vitro* and *in vivo*. Cell Death and Differentiation 12, pp. 805-814

Sabatino, M. A., Previdi S., Broggin M. (2007) In vivo evaluation of the role of DNp73 α protein in regulating the p53-dependent apoptotic pathway after treatment with cytotoxic drugs. International Journal of Cancer 120(3), pp. 506-513

Publications by the candidate on topics related to the work described, but published

before starting PhD

Sabatino, M.A., Colombo, T., Geroni, C., Marchini, S., Broggin, M. (2003)

Enhancement of *in vivo* Antitumor Activity of Classical Anticancer Agents by Combination with the New, Glutathione-Interacting DNA Minor Groove-Binder, Brostallicin. Clinical Cancer Research 9, pp.5402-5408

Posters presented at international meetings by the candidate emanating from the

work described in this thesis

M.A. Sabatino, C.Geroni, M.Broggin

"Antitumor activity of brostallicin on human prostatic cancer cells: fundamental role of combination with hypomethylating agents"

EORTC-NCI-AACR SYMPOSIUM "Molecular Targets and Cancer Therapeutics"
Geneva, Switzerland, September 28th – October 1st 2004

M. Broggin, **M.A. Sabatino**, C.Geroni, S.W. Weitman

"Antitumor activity of brostallicin on human prostatic cancer: role of combination with hypomethylating agents"

EORTC-NCI-AACR SYMPOSIUM "Molecular Targets and Cancer Therapeutics",
October 22-26, 2007, San Francisco, California